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BIOLOGY OF THE PLEUROPNEUMONIALIKE ORGANISMS

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BIOLOGY OF THE PLEUROPNEUMONIALIKE ORGANISMS*

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INTRODUCTION

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In the past few years I have realized from private conversations and correpondence that there has been an increasing interest in the pleuropneumonia roup of organisms. The recent phase of intensive study has been prompted y the association of the organisms with respiratory infections of poultry, for uch infections are of enormous economic importance. Until recently these rganisms have represented one of the most neglected groups in microbiology nd it is worth recalling the various phases in the study of this group.

The agent responsible for contagious bovine pleuropneumonia was first ecognized as a filterable organism and therefore was included among the viruses ntil later, when it was cultivated on an artificial medium. The subsequent solations of pleuropneumonialike organisms from mice and rats were made by irologists studying the effects of viruses in these animals and, in so doing, ncountering them as contaminants. At the beginning, therefore, investiators tended to study these organisms chiefly by methods employed in virology, eglecting to observe such factors as cultural behavior and metabolism. It s worth noting that, in a monograph concerning agalactia published in 1951, he organism was referred to everywhere as a virus. Moreover, the organism vas studied as a virus, without any description of its cultural behavior. This vas in 1951, although the organism was first isolated in 1923.

The one aspect of these organisms that has been studied intensively is their norphology. Only comparatively recently has it been appreciated that the rganisms possess other interesting features, apart from their peculiar morhology, that well repay study. Therefore, in recent years bacteriologists nd biochemists have begun an investigation of their physiology and metablism.

However, for a long time in the medical bacteriological field there has been tendency to regard these organisms as something very peculiar and unimortant; in fact, their existence has almost been doubted through a failure to nderstand the significance of references to "L forms" and "L organisms." nly the veterinary bacteriologist knew much about them and appreciated heir importance, in fact, only the veterinarian working in certain parts of the orld, notably Africa. In this monograph, S. E. Piercy explains the pathogenic nportance of the organism of contagious bovine pleuropneumonia in certain arts of the world.

I have never liked the term "pleuropneumonialike organisms," for it suggests distinction between the organism of bovine pleuropneumonia and the pleuroneumonialike organisms. As there is no distinction in the properties of these rganisms, I have preferred to speak of "organisms of the pleuropneumonia roup." The use of the term "L organism" has caused a tremendous amount of onfusion, particularly to those not well acquainted with this field. One of the roblems treated in this monograph is that of nomenclature. E. A. Freundt nd I have published a suggested nomenclature and classification.[1] These proposals are tentative, but I hope that they can form a basis for discussion and I hope that we can come to some sort of agreement on them. It seems necessary to emphasize the fact that, since these organisms are capable of cultivation in artificial media, they can be studied by the methods available for bacteria. Our methods of biological study are limited at the moment and sorely need extending. In bacteriology it is standard practice to identify any organism that is isolated by determining its cultural and serologic properties. The identity of any organism is checked against representative strains in type collections and by the use of standard antisera. It seems to me that this should become routine practice in dealing with organisms of the pleuropneumonia group; this objective can be achieved by recognizing named species.

It is true that, if we are to classify these organisms in a taxonomic system, we must establish their relationship to bacteria and other microorganisms. This matter also is discussed in this monograph. This group of organisms possesses many interesting properties that merit study for academic reasons alone. Of particular interest is their relationship to other groups of microorganisms. First, there is their relationship to bacteria through the L-phase organisms. Like viruses, these are filterable, for their smallest viable elements are of the same size as the larger true viruses. Their inhibition of growth by antisera bears some resemblance to the neutralization of a virus. In regard to their metabolism, there is a resemblance to protozoa, for they require sterols. Sterols are not needed by bacteria, but certain protozoa require them. These relationships are considered in these pages. It is notable that the morphology of the pleuropneumonia group was first described in 1910, but even now there is still no agreement on morphology. I hope that this monograph will contribute to greater agreement among morphologists.

I have mentioned some of the academic problems studied; on the practical side it seems to me that particular attention should be given to two aspects. The first is pathogenicity. Some of the organisms are most definitely pathogens: for instance, the organism of bovine pleuropneumonia. However, even here, as E. Klieneberger-Nobel points out, pathogenicity is not a straightforward matter, for it is difficult to reproduce the natural disease. At the other extreme, certain species are clearly saprophytes or, more commonly, commensals. Between these two extremes, however, there is a number of organisms whose pathogenic significance is very difficult to determine. There are the organisms of the bovine genital tract. Do they cause infection? Do they predispose to infertility? Is chronic respiratory disease of chickens due to pleuropneumonialike organisms? In the human field, what is the cause of nongonococcal urethritis? Is it due to the pleuropneumonialike organisms that can be isolated from a large proportion of cases? Despite considerable study by numerous investigators, these questions remain unanswered. This is particularly true in the case of human infections. The problems of determining the pathogenicity of organisms of the pleuropneumonia group appear to be more difficult than those of bacteria. It is often impossible to fulfill Robert Koch's postulates. It may be that with some organisms pathogenicity of a strain is rapidly lost once they are in artificial cultures. It may be that pathogenicity could be maintained if the right constituents for the medium were known. It is possible that tissue culture may be a useful tool in investigating pathogenicity.

Secondly, attention should be devoted to growth requirements, with a view to improving media. Many investigators have encountered strains that are difficult to establish on artificial media, even though such media grow stock strains well. The organisms of the pleuropneumonia group inhabiting the bovine genital tract provide an example. When the semen of a bull was cultured on the ordinary medium, cultures were completely sterile, but there was a profuse growth in the same medium after the addition of mucin or thymic nucleic acid. Are there in existence members of this group that, in fact, are unable to grow on any media at present in use? In other words, are there diseases caused by this group of organisms of which we are as yet ignorant, because we are unable to cultivate the organisms in artificial media? It seems to me, therefore, that studies of the physiology and metabolism of the organisms may produce results of practical value.

There is, perhaps, a third practical problem. How can we protect against such infections due to organisms of the pleuropneumonia group and how can these infections be treated? The methods for preventing contagious bovine pleuropneumonia itself are still far from satisfactory.

In my opinion these organisms are much easier to study than is generally realized. Their biological and serologic properties may be examined by the same methods as those used for bacteria, provided that certain modifications in technique are made, as in methods of inoculation and incubation. The usual bacteriological principles must be followed, as in examining strains isolated from single colonies. The colonial appearances are of the greatest importance in identification and these may be seen with a dissecting microscope, using ×10 magnification. Higher magnification is required only when morphology is studied. It is essential to use a reliable medium for cultivation. However, if bacteriostatics such as thallium acetate and penicillin are added to the medium, there is much less difficulty with contamination by other organisms than is encountered in studying bacteria or in growing viruses in tissue culture. The main risk of contamination is that of contaminating one strain of pleuropneumonialike organism with another; great care is necessary to prevent this. The identity of a strain maintained by continued subculture must be checked continually.

Reference

1. Edward, D. G. ff. & E. A. Freundt. 1956. The classification and nomenclature of organisms of the pleuropneumonia group. J. Gen. Microbiol. **14:** 197.

Part I. Morphology and Classification

MORPHOLOGY AND CLASSIFICATION OF THE PPLO

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It is interesting and surprising that since the detection, in 1898, of the first member of the pleuropneumonia group of organisms (PPLO), no less than about 40 papers dealing chiefly with the morphology of these organisms have appeared. I believe that the number of morphologic studies of very few other microorganisms even approaches this total.

It might be felt that the lively interest thus far shown in the morphology and mode of growth of the PPLO is highly exaggerated. While it is easy to realize the fundamental importance of morphologic studies in comparative zoology and botany, it may be argued that the relatively limited morphologic variations found within the bacteria and related microorganisms can help the taxonomist to create a classification system only along broad lines. However, even with this reservation it should still be kept in mind that the morphologic characteristics of the individual microorganisms and of their cell communities have been a valuable guide since the early days of microbiology both in everyday diagnostic work and in any approach to a deeper understanding of the phylogenetic relationships between microorganisms. Moreover, the significance of these characteristics greatly increases with increasing morphologic differentiation and evolution of the organisms.

In fact, the existence of the large number of papers discussing the morphology of PPLO reflects the state of confusion and disagreement prevailing through many years in this field. My paper includes two main points: the question of whether the organisms are mycelial in structure, and the problem of the significance of the "large bodies" in the developmental cycle of the organisms.

As a basis for further discussion, I shall give first a description of what seems to me to represent the normal morphologic development of the type species of the group, the organism of bovine pleuropneumonia, *Mycoplasma mycoides* (Freundt, 1955). The description is based on observations of living cultures in the ordinary light and phase-contrast microscopes, and on electron micrograph studies (Freundt, 1952*a*, 1954, 1957, 1958).

The starting point of growth is the elementary body, the size of which is about 250 to 300 mμ, and which extrudes one or more exceedingly thin, optically homogeneous filaments. The filaments always terminate in a tiny, strongly refractile, club-shaped, or spherical body of the same order of size as the elementary body. This terminal body functions as the growth center for the development of forked pairs of new filaments, resulting in the formation of dichotomously branching structures. In addition, however, a true lateral branching, characterized by the apparently coenocytic outgrowth of lateral branches from the stem filament, occurs fairly frequently. As a result, a single-celled mycelium has developed during the first 12 to 18 hours of growth (FIGURES 1, 2, and 3).

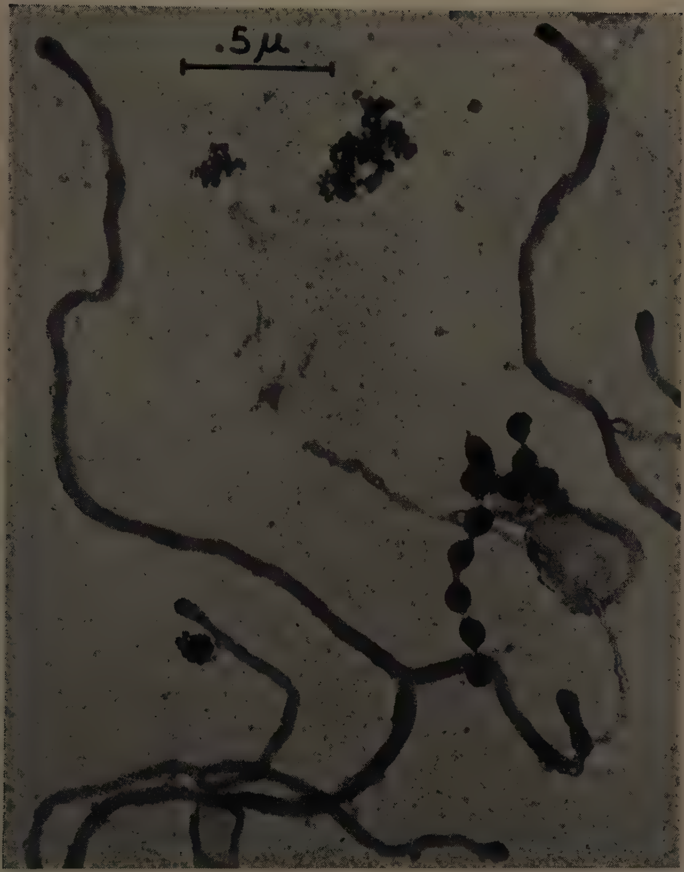

FIGURE 1. Electron micrograph of *Mycoplasma mycoides* var. *mycoides* from a 24-hour
erum-broth culture showing branching filaments and a filament with endomycelial ele-
nentary bodies. Electron fixation (see Freundt, 1952*a*) followed by fixation for 5 min. with
he vapors from a 2 per cent solution of osmium tetroxide.

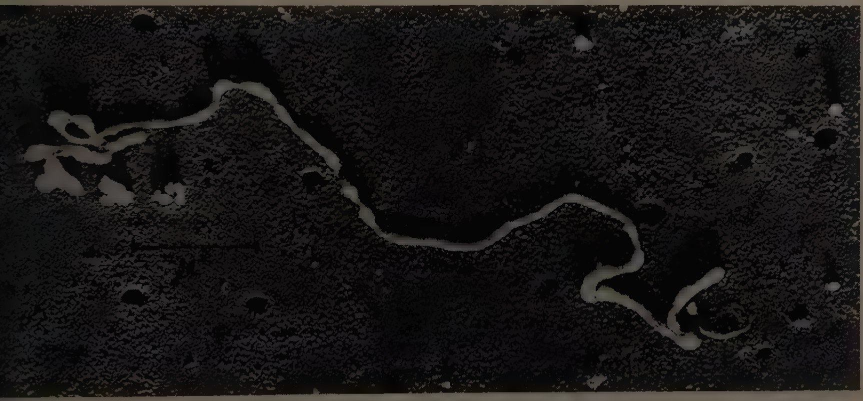

FIGURE 2. Electron micrograph of palladium-shadowed filament of *M. mycoides* var.
yc. from a 24-hour serum-broth culture. Electron fixation.

The next stage of growth is that of chain formation, which is initiated by the appearance in the interior of the filaments of numerous closely set, regularly spaced, and strongly refractile spherical corpuscles of uniform size and shape. This developmental process is not associated with any visible formation of transverse septa and, in the beginning, the filament retains its uniform cylindrical caliber. Very soon, however, distinct constrictions are seen at the intervals between the spheres, dividing up the filament into a regular chain of coccoid elements (FIGURE 1). The whole process apparently may occur simultaneously throughout the entire length of a filament, or it may be confined for some time to smaller or longer parts of the filament. The originally single-celled mycelium has now passed into a multicellular stage, since the spherical elements are actually new young elementary bodies that are subsequently liberated through a fairly rapid disintegration of the chains and are able to repeat the whole life cycle when transferred to a fresh medium.

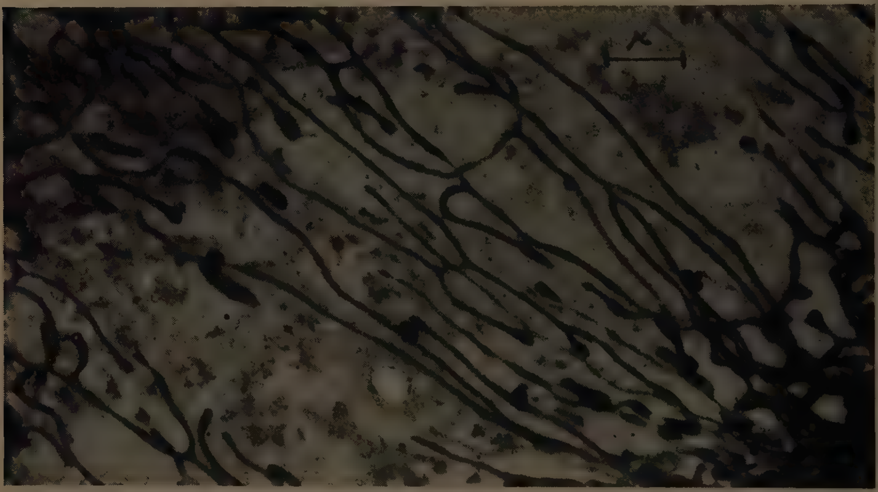

FIGURE 3. Electron micrograph of *M. mycoides* var. *myc.* from a 24-hour culture grown on a collodion film floating on serum broth, showing branching filaments. No fixation.

The micrographs chosen to illustrate the growth phases were prepared from broth cultures. On looking at a colony on solid medium, it is hardly possible, even under the strongest high-power lens, to find the slightest resemblance to the structures seen in the broth cultures. Apparently, the colony consists only of a heap of granular or coccobacillary elements growing down into the depth of the medium and, possibly, surrounded by the well-known flat peripheral zone. It is possible, however, by means of a very simple technique devised by Ørskov (1927, 1942) to demonstrate the same sequence of events as those observed in broth cultures. One- to 2-day-old cultures should be chosen for the study, and the colonies should be of the uniformly small granular type, showing no signs of passing into the stage characterized by development of large bodies and the production of extracellular substances.

A square of agar supporting an appropriate number of colonies is cut out; a ew cotton fibers and a drop of tap water or broth are placed on the surface of he culture. After covering with a coverslip, the preparation is left at room emperature or in the incubator for 15 to 30 min. On subsequent inspection under the oil-immersion lens, a radical change may now be noticed, particuarly if colonies that lie near or are crossed by one of the cotton fibers are selected. Fairly long filaments, frequently in enormous numbers, may be seen emerging from the surface and the marginal part of the colony and, being in a state of constantly undulating movement produced by the fluid currents and the Brownian effect, present a fascinating sight. Usually the PPLO filaments run parallel to the cotton fiber, as their orientation in the preparation is determined by the direction of the current arising in the supernatant fluid. In other colonies, which do not lie in direct association with a cotton fiber, a halo of mycelial filaments spreading radially from the entire circumference of the colony sometimes may be observed (Figure 2, in Freundt, 1958). According to the age of the colony, the branching filaments may be homogeneous and firmly attached to the central part of the colony, or they may appear as chains of elementary bodies, smaller and larger fragments of which are easily torn off from their attachment to the colony. It should be mentioned here, however, that it is by no means always easy to demonstrate the mycelial structure of every colony. The proportion of colonies that present, in a given culture, the typical mycelial appearance depends on the composition of the medium and other variable growth conditions. I shall revert to this point later.

It is not difficult to explain what happens to the colonies when Ørskov's technique is used. In the intact dry colony the filaments avoid demonstration apparently because they form a closely tangled mass. When a thin layer of fluid together with a few cotton fibers are applied on the surface of the culture, fluid currents will arise along the cotton fibers due to the pressure of the coverslip. Actually, these currents help the originally matted filaments to unfold n the supernatant broth layer. I am aware, of course, that another explanaion may be given, and I shall discuss that in due time.

FIGURE 3 shows the growth of the pleuropneumonia organism under experinental conditions that may be compared, albeit with some reservation, to the rowth on solid medium. In this case the organisms were grown on the surface f a collodion film kept floating on serum broth, a technique indicated by Hillier *et al.* (1948) for the electron microscopic study of growth supported and imited by a surface. Here again the long-filamented branching structures will e seen.

Naturally, it is highly important to decide whether the description of the ype species as a mycelial organism that reproduces through the formation nd subsequent release of endomycelial elementary bodies can be extended to he other members of the group. From my experience, based on studies of a onsiderable number of strains representing the majority of all known species, his question must be answered in the affirmative. On the other hand, it should e stressed at once that with many strains it is considerably more difficult to lemonstrate the mycelial structure than it is with the bovine pleuropneumonia organism. The filaments are generally notably shorter in other species, and the single-celled mycelial phase of growth is frequently transitory and unstable, the short filaments soon breaking up into individual coccoid elements. In this connection it might be mentioned that this situation is by no means unique for the PPLO, since a similar range of variation as regards the degree of the development and stability of the mycelium is found within another large group of mycelial organisms, the actinomycetes.

To illustrate the claim of morphologic identity between the type species and other species of the group, a few electron micrographs are presented of human PPLO, *M. hominis* and *M. fermentans*, and of the saprophytic *M. laidlawii* (FIGURES 4 and 5). It is seen clearly, in particular from the micrographs of *M. laidlawii*, that many of the filamentous forms are so very small that they would hardly be discernible, if at all, in the light microscope. It is no wonder, therefore, that it has proved difficult to recognize the mycelial structure of the small mycelial strains.

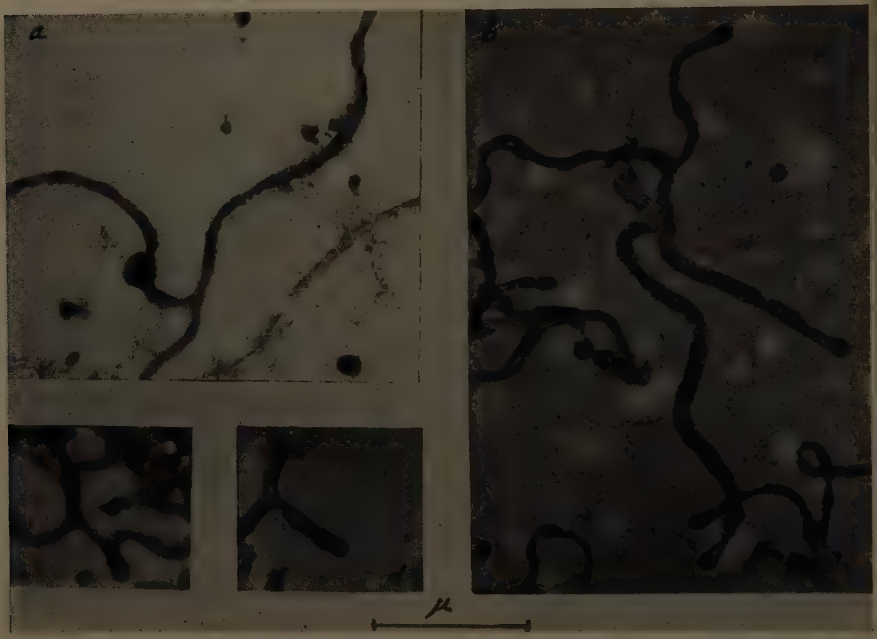

FIGURE 4. Electron micrographs of human PPLO from 24- to 42-hour ascitic-broth cultures, showing branching filaments. Electron fixation. Key: *a* and *d*, *M. hominis*; *b* and *c*, *M. fermentans*.

The description I have given of the morphology of the PPLO agrees fairly well with observations made by other investigators, both with the bovine pleuropneumonia organism and with several other species. Actually, the first clear and detailed description of the morphologic development of *M. mycoides*, based on studies of growing cultures in the living state on solid medium, was given by Ørskov in 1927 and in subsequent studies (1938, 1942).

Moreover, I might mention in this connection authors such as Nowak (1929), Wróblewski (1931), Ledingham (1933), Tang *et al.* (1935), Preston (1942), Beveridge (1943), and—from recent years—White *et al.* (1954) and Bartmann and Höpken (1955), who are all in essential agreement with the description presented here. Therefore, it would have been unnecessary to discuss this problem again, were it not for the fact that the classic conception of the morphology of the PPLO, at least as regards its validity for the group as a whole, is still seriously questioned or frankly rejected by prominent and experienced workers in this field.

First of all, it is maintained, for example by Klieneberger and Smiles (1942), Klieneberger-Nobel (1954), Dienes (1945, 1953), and Liebermeister (1953), that the branching filamentous structures do not represent the natural forms of the organisms. These filaments are considered chiefly as artifacts, in the ense that they have been produced during the preparation by the action of external physical forces (such as fluid currents or the pressure of the coverslip) on morphologically labile organisms, the plasticity of which can be ascribed o their lack of a firm cell wall. In other words, the branched filaments are upposed to be chance manifestations of ameboid cells that can assume practically any shape according to circumstances.

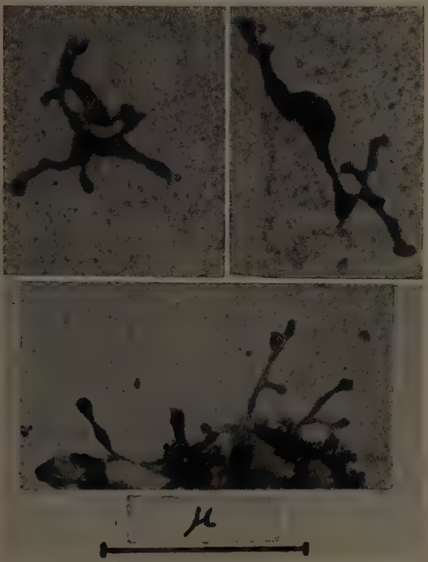

FIGURE 5. Electron micrographs of *M. laidlawii*, Type A, from 18-hour ascitic-broth ultures, showing short mycelia. Electron fixation.

Because of the direct observations of growing cultures I have made, it is very difficult to accept the validity of this interpretation. Moreover, the filaments demonstrated in the electron micrographs do not give the immediate mpression of having developed by chance; on the contrary, they would appear o be well-defined cellular structures of characteristic shape, passing through a regular series of distinct developmental stages—close parallels to which are known, moreover, from other mycelial organisms such as the actinomycetes. I am prepared to admit that single nonbranching filaments might be the occasional result of traction on a spherical cell. However, how could repeatedly branching filaments develop through the action of, for example, a fluid current acting chiefly in one direction? The development of long chains of uniform elementary bodies, also occurring in branching configurations, is perhaps even more difficult to explain as the result of external forces. Altogether, such an explanation would seem to be farfetched and to lack reasonable basis, since it would apparently mean that it is possible, simply by means of technical errors in methods of preparation, to imitate nature as manifested in the highly differentiated morphology of the actinomycetes.

In addition to these considerations, previously I have tried to provide experimental support for the conception that the formation of branching mycelia does represent the normal mode of growth of the PPLO (Freundt, 1952*a*). This was done by studying the influence of various growth conditions on the morphology, the supposition being that optimal growth conditions are likely to favor a normal morphologic development, whereas poor or harmful conditions might be supposed to stimulate the development of aberrant and involutionary morphologic features.

As an example, I can refer to experiments made with cholesterol, which is a growth factor important to the PPLO. *M. mycoides* was cultivated on solid medium enriched with a type of ascitic fluid, the growth-promoting property of which was in itself relatively poor. Cholesterol was added in concentrations of from 0.1 to 0.5 mg./ml., and the cultures were read after 48 hours' incubation. The influence on growth of an increasing concentration of cholesterol was indicated by a definite increase of the average size of the colonies, followed at the highest concentrations by a slight decrease. It should be emphasized that the measurements of the colony diameter were made under a high-power dry lens on the intact colonies, that is, before the application of broth and coverslip to the surface of the culture. A subsequent study of the colonies by means of Ørskov's technique showed the occurrence of 3 types of colonies: (1) Type-A colonies distinguished by well-developed, long-filamented mycelia; (2) Type-B colonies with poorly developed mycelia, only few and short filaments being demonstrable; and (3) intermediate forms. On the medium without cholesterol all colonies were Type B, with poorly developed mycelia. The addition of 0.1 mg. of cholesterol/ml. resulted in the growth of a low proportion of typical mycelial colonies, while these colonies clearly predominated on the media with higher concentrations of cholesterol, a maximum of about 90 per cent of the total number of colonies being obtained on the medium containing 0.3 mg. of added cholesterol per ml. It also appeared from the experiments that, on the whole, there was a fair parallelism between the ability of cholesterol to stimulate growth as such (as indicated by the size of the colonies) and its influence on the morphology. A similar dependence on the conditions of cultivation was observed in experiments with media enriched with different kinds of serum. For example, the poor growth characterizing *M. mycoides* on media enriched with rabbit serum, which is remarkably poor in cholesterol, was always associated with a sparse development of the mycelium. On the other hand, the incorporation in the medium of human erum, distinguished by a high level of cholesterol, invariably resulted in both heavy growth of big colonies and the development of numerous long filaments n the majority of the colonies. Horse and ox sera take an intermediate position between rabbit and human serum in respect to both their content of cholesterol and to their influence on the growth and morphology of *M. mycoides*.

The dependence of maximal mycelial development on optimal growth conditions would seem to be another strong argument in favor of the conception that his mode of growth is the normal and typical one: if a tree is planted in a deficient, sandy soil and in bad climate, the result probably will be only a small ree with short crooked branches, one that can hardly be expected to produce much fruit. On the other hand, were the development of the filaments merely an artificial phenomenon, they might be supposed to appear quite as easily under poor growth conditions as on an optimal medium.

There still remains for discussion the significance of the large bodies, pleomorphic elements that, in fluid medium, take the shape of regular spheres while, on solid medium, usually are flat and with irregular contours. According o their age, large bodies may contain a fairly dense, homogenous cytoplasm; hey may be filled with tiny granules; or they may even appear as empty vesicles. Most of the large bodies are apparently swollen elementary bodies, while others appear to be fat droplets resulting from the lipid metabolism of he organisms. The tendency to develop large bodies varies to some extent with different strains. Generally these large bodies are particularly frequent n old cultures although, during growth on poor media, they sometimes appear at an early stage of growth. On solid medium, their number is usually inversely proportional to the number of visible mycelial elements. Thus they often predominate in the Type B colonies, and it follows from what has already been aid that they are probably aberrant forms, although this conception is not hared by all investigators.

Further attempts to elucidate the nature of the large bodies were previously made by me in experiments with *M. mycoides* grown in media with different oncentrations of glucose, which is attacked by this species with the production f acid (Freundt, 1952*b*). In serum broth with a low glucose content and a orrespondingly slight decrease of the *p*H of the medium during growth, the morphologic development ran through the stages described for the normal rowth cycle and without the appearance at any time of true large bodies. At the same time the proportion of viable elements in the culture was maintained at a relatively high level for weeks.

In the medium with a higher concentration of glucose, the initial growth of he mycelium was identical with that found in the former experiment. However, the fall of the *p*H below 6 was associated with an increasing swelling of he elementary bodies into large bodies, which later became granulated and acuolized and soon completely dominated the morphologic appearance of the ulture. Concomitantly, the content of elements capable of germinating in ubcultures rapidly decreased. It was shown further that the development of arge bodies in this system could be prevented by continuous neutralization of the acids produced by the breakdown of glucose. Apparently the development of the large bodies from the elementary bodies was due to damage of their cell walls brought about by the acids and resulting in a lowered osmotic resistance of the cells. Actually, a lowered osmotic resistance was demonstrable, as shown in supplementary experiments, prior to the spontaneous swelling of the elementary bodies. The fact that the large bodies that developed in acid medium could not be propagated in subcultures is, of course, no proof in itself that they are always nonviable. However, as mentioned previously, the relative inability of the large bodies to reproduce is in my experience a constant characteristic of these elements, and one which does not depend on the accidental conditions during which they develop. In conclusion, there seems to be little doubt that the large bodies should be regarded as abnormal, involutionary forms that probably do not deserve the interest frequently shown in them.

There are three main aspects of the significance of the morphology of the PPLO to their classification that should be considered.

(1) *Identification on morphologic grounds of any newly isolated microorganism as a PPLO.* The typical gross appearance of the colony is in itself a valuable guidance, although—particularly in the hands of less experienced workers—it may lead to errors due to confusion with the L-phase variants of bacteria. Unfortunately, the practical use that can be made at present of the cellular morphology in the identification of the PPLO is limited by the difficulties involved in the recognition of their mycelial structure. However, although it is obviously difficult to obtain general agreement on this point, it would be advisable not to base the classification of an organism as a PPLO merely on the grounds of peculiar pleomorphic features. The adherence to such criteria in several cases has led actually to erroneous classifications.

(2) *Subdivision of the group of PPLO into morphologic subgroups.* In previous papers I suggested the establishment of three morphologic groups of PPLO, based on differences in the length of the filaments, the frequency of branching, and the stability of the mycelium (Freundt, 1954, 1958). It follows that, in my opinion, there is no morphologic difference in kind, but only one of degree, between different strains of PPLO. Consequently, the possible establishment of taxonomic subgroups, such as different genera, cannot be based exclusively on morphologic criteria. The taxonomic situation in this respect perhaps is illustrated best by the fact that the bovine variety of the type species, *M. mycoides*, is distinguished by its particularly richly developed, long-filamented mycelium, while the strains of the closely related caprine variety of the same species, which I examined, were definitely short-filamented.

It may be mentioned in this connection that in recent years there has been a tendency among morphologists to acknowledge that *M. mycoides*, *M. agalactiae* and, possibly, a few other species might be defined fairly well as mycelial organisms while, on the other hand, the validity of this characterization is not recognized for the majority of the PPLO strains. Were this true, there would be, insofar as I can see, only one logical conclusion to be drawn: to realize that the PPLO do not constitute a well-defined taxonomic unity, since mycelial organisms cannot be classified together with definitely nonmycelial organisms.

(3) *Taxonomic position of the PPLO in relation to other microorganisms.* In his respect, particular attention should be paid to the possible relation between PLO and the L-phase variants of bacteria. The claim of a close morphologic imilarity on the cellular level between the two groups of organisms is based artly on the occurrence in both of them of pleomorphic large bodies and on he belief that the latter are able to reproduce through the liberation of intra-ellular granules. According to the views adduced by me and certain other nvestigators on the entirely involutionary nature of these elements, no im-ortance can be attached to them in the establishment of taxonomic relation-hips. Altogether, the morphology of the L-phase variants is in my opinion undamentally different from that of the PPLO, since the development of ranching, elementary body-forming filaments is by no means a common haracteristic of the L phase.

In recent years particular interest has been shown in the apparently similar ature of the cell envelope in PPLO and in L-phase variants, both of them pparently lacking a firm cell wall similar to that found in ordinary bacteria. n collaboration with Birch-Andersen, I recently started some electron mi-roscopic studies of ultrathin sections of the rapidly growing caprine variety f *M. mycoides*. The cultures chosen for our preliminary studies were in the erminal growth phase, that is, comprising exclusively free elementary bodies. Thereby, obtaining a sufficient amount of material for the preparations was onsiderably facilitated. The basic methods were essentially identical with hose used by Thorsson and Weibull (1958) in a recent study of protoplasts nd L forms, although various modifications of their technique were included n our experiments. The fixation of the organisms was made directly in the luid growth medium with 0.8 per cent of formaldehyde and with the vapors rom a 1 per cent solution of osmium tetroxide (2 hours at 37° C.). The results vere very similar whether each of these methods was used separately, or vhether they were combined, with the exception that the general contrast vas increased somewhat with osmium fixation. On the other hand, the mate-ial used for the embedding had a decisive influence on the appearance of the rganisms. FIGURE 6 shows sectioned elementary bodies embedded in methyl nethacrylate as used by Thorsson and Weibull. It will be seen that most of he cells are merely swollen "ghosts," the internal structure and outer limits f which are severely damaged. The results obtained with the epoxy resin Araldite* are illustrated by FIGURE 7, showing elementary bodies with a dense, ather uniformly granulated cytoplasm surrounded by a single, extremely lelicate cytoplasmic membrane. The superiority of Araldite to methacrylate s embedding material observed in the present experiments is in complete greement with the experience previously obtained with ordinary bacteria Maaløe and Birch-Andersen, 1956; Glauert and Glauert, 1958; Birch-Andersen, 959). Thus far we have made no comparative studies with ultrathin sections f L forms. Thorsson and Weibull demonstrated a similar thin cytoplasmic nembrane in the L phase of *Proteus*. On the other hand, the internal structure f the L forms is apparently rather different from that of PPLO, but direct omparison is difficult, because the work of Thorsson and Weibull was based exclusively on embedding in methacrylate and because most of their micrographs, in contrast to ours, obviously represent cells in the large-body stage. However, further studies in this field will very probably provide interesting information.

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* Product of Ciba (Aero Research Ltd.), Duxford, Cambridge, England.

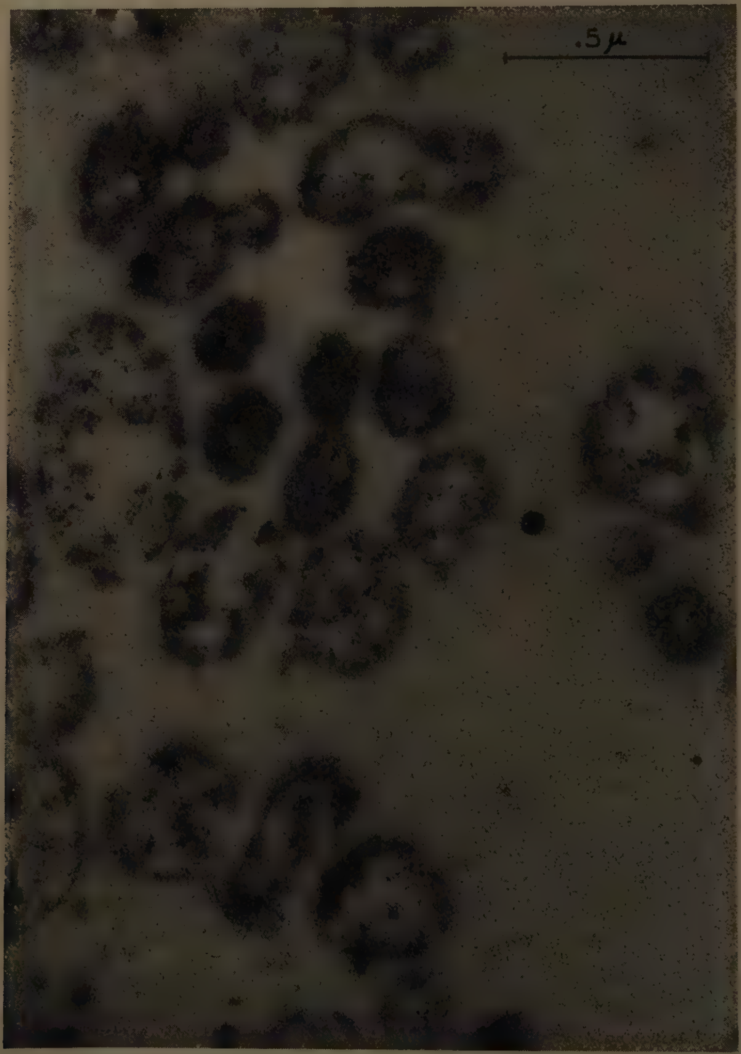

Figure 6. Electron micrograph of ultrathin section of *M. mycoides* var. *capri* from an 18-hour broth culture, showing "ghosts" of elementary bodies. Fixed with formaldehyde and osmium tetroxide vapors; embedded in methacrylate.

Before leaving the discussion of the PPLO and the L forms, I shall cite the fact that the belief in a close relationship has been formulated in different ways. When Klieneberger (1935, 1936) first isolated the L1 from *Streptobacillus moniliformis*, she thought that L1 was a true PPLO living in natural

mbiosis with the streptobacilli. In recent years it has been held by some
orkers that the PPLO are stabilized L-phase variants of bacteria similar to
ie L-type colonies of known bacterial origin. However, there would seem
be a third possibility that, to my knowledge, has not been considered thus
r, one that would not invalidate the conception of the PPLO as an autono-
ous taxonomic entity: that is, the possibility that true mycelial PPLO may
evelop L-phase variants. The abnormal PPLO colonies described by me as

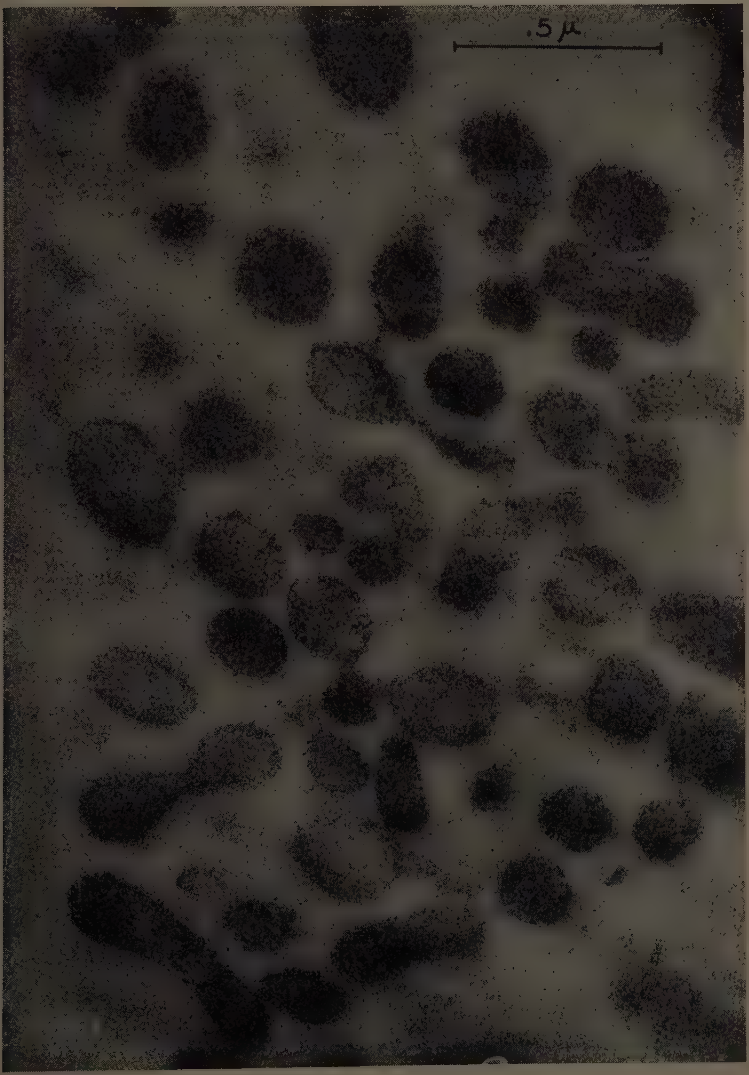

FIGURE 7. Electron micrograph of ultrathin section of *M. mycoides* var. *capri* from an
-hour broth culture, showing elementary bodies with dense cytoplasm and delicate cyto-
smic membrane. Fixation as in FIGURE 6, but embedded in Araldite.

Type B and characterized by the development of large bodies at the expense of the mycelial elements perhaps might be considered as L-phase colonies of the PPLO.

As to the relationship between PPLO and bacteria in general, it is clear that the recognition of the mycelial nature of the PPLO, together with their reproduction through the formation and subsequent liberation of endomycelial elementary bodies will be sufficient in itself to place them as a separate and distinct taxonomic group. Although similar structural and reproductive features are found in the actinomycetes, these differ from the PPLO both cytologically and in other respects. For this and other reasons the PPLO were classified by Edward and Freundt (1956) as a separate order. On the other hand, in view of the pronounced heterogenicity already characterizing the vast group of microorganisms conventionally classified as bacteria (class Schizomycetes Nägeli) there would be no reason, insofar as I can see, for the establishment of a separate class for the PPLO.

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MORPHOLOGY OF THE PPLO AND L FORMS OF *PROTEUS**

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Dienes[1,2] has characterized the so-called L organisms in the cultures of *Streptobacillus moniliformis* described by Klieneberger[3] as variants of the bacterial form. Dawson and Hobby,[4] as well as other investigators,[5-7] support this view. Subsequently, Dienes demonstrated the L form with other bacteria without the aid of penicillin and, after 1947, sometimes with the aid of penicillin.[8] In contrast, until 1949 Klieneberger considered the L-form phenomena to be a symbiosis of organisms of the pleuropneumonia group with bacteria.[9]

This development shows that a morphologic relation between the L form and the pleuropneumonia group exists. Therefore, it is of interest to compare the results obtained by modern methods of examination: that is, with the electron and the phase microscope, as well as with special methods of preparation.

PLEUROPNEUMONIALIKE ORGANISMS (PPLO)

Most of the references to PPLO make it obvious that no clear conception of the morphology and the multiplication of these organisms has been achieved. Very different shapes and complicated and partly very queer cycles of multiplication have been described.[10-27] The reason for this is probably the great difference of size of the cells and the difficulty of properly observing these organisms because (1) the smallest forms lie on the limit of visibility of the ordinary microscope and (2), the organisms are easily deformed due to their fragility and softness. Although the latter fact is well known, most investigators use methods of preparation that include the possibility of damage to the organisms. Even the careful placing of a coverslip on a colony on the agar surface causes considerable adhesion and initiates currents that deform the organisms (SCHEME 1). An even greater mutilation is caused by centrifuging, by taking up the sediment and transferring it to another medium, and by letting this suspension dry onto the electron microscope film. Only certain methods are suitable for investigation of these organisms. It is necessary to examine carefully whether the method of preparation has caused any deformation or change of structure. This examination is easier if the organisms are examined in their natural growth surroundings, and if the batch of preparations is examined by various methods and enlargements and the results of the optical- and the electron-microscope examinations are compared. In this way one obtains a more reliable picture of those batches in which the highly sensitive organisms have been deformed by external influences. FIGURE 1*a* is a picture taken with an ordinary microscope of a nondeformed colony of PPLO with elliptic to roundish and regular forms. FIGURE 1*b* shows a similar colony distorted in one direction. FIGURE 1*c* shows the border of a nondeformed colony, and FIGURE 1*d* illustrates a similar site in a different preparation with

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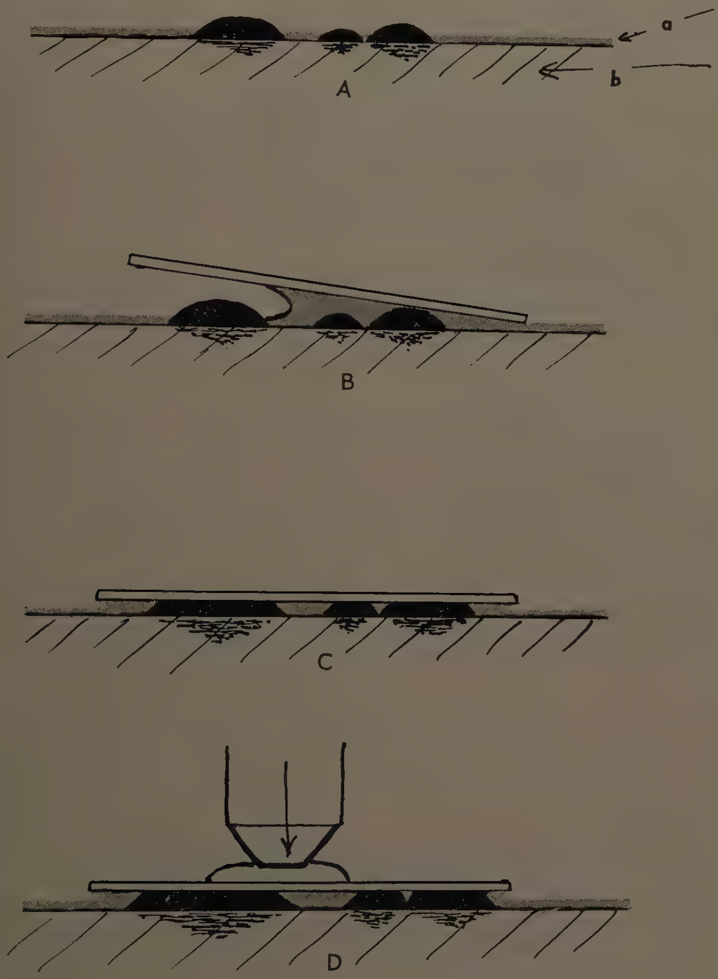
organisms pushed out of their natural growth surroundings. If such a faulty preparation is examined in the electron microscope, myceliumlike threads and string forms (partly with terminal, partly with median swellings and irregular polygonal structures, as frequently described in literature) are found. FIGURE 1*e* and *f* show the organisms of pleuropneumonia of cattle: elements with long thin threads and protozoalike or spermatozoalike forms. FIGURE 1*g* shows the Laidlaw-Elford strain A. Here are seen some tailed bodies displaced from their former position and leaving a thin, mucous trace behind, in addition to round and elliptic forms.



SCHEME 1. (A) Colonies on agar surface: *arrow a,* layer of liquid; *arrow b,* agar. (B) Mounting a coverslip; tensions in the layer of liquid. (C) Coverslip lies on colonies and agar surface. (D) Microscopic observation; the pressure of the objective is transferred on coverslip by the oil.

Preparations of undistorted colonies show organisms with the following rather simple morphology: elliptic disks or bubble-shaped forms that give different, nonhomogeneous contrasts and great variance in size. A cell wall as seen in bacteria cannot be found. FIGURE 2*a* to *c* shows the organisms responsible for cattle pleuropneumonia; FIGURE 2*d* to *g* shows microcolonies of a Laidlaw-Elford strain. Agar fixation on collodion film[28] was used for the

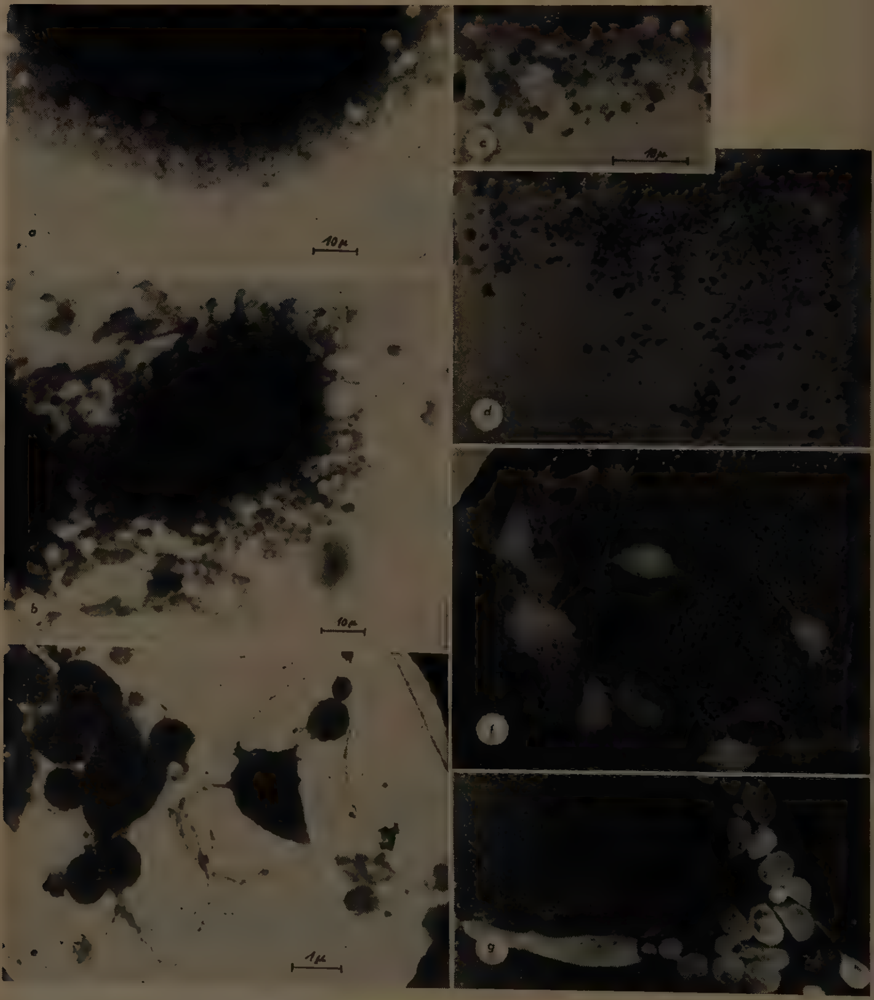


FIGURE 1. (*a*) Colony of pleuropneumonia organisms of cattle; 48 hours; undistorted cells; ordinary micrograph; methylene blue. (*b*) Colony of pleuropneumonia organisms of cattle; 48 hours; generally distorted in one direction; ordinary micrograph; methylene blue. (*c*) Margin zone of a colony as in *a*; undistorted; agar fixation, Giemsa; ordinary micrograph. (*d*) Margin zone of a colony as in *b*; distorted; spermatozoa- and mycelialike forms; agar fixation, Giemsa. (*e* and *f*) The same preparation as in *d*, but under the electron microscope; *f*, shadowed. (*g*) PPLO Laidlaw-Elford strain A; some cells displaced, leaving behind long filaments resembling spoor; electron micrograph, shadowed.

preparation of the cells of FIGURE 2*a* to *c*; agar filtration after the method of Kellenberger[29] was employed for the preparation of the cells shown in FIGURE 2*d* to *g*.

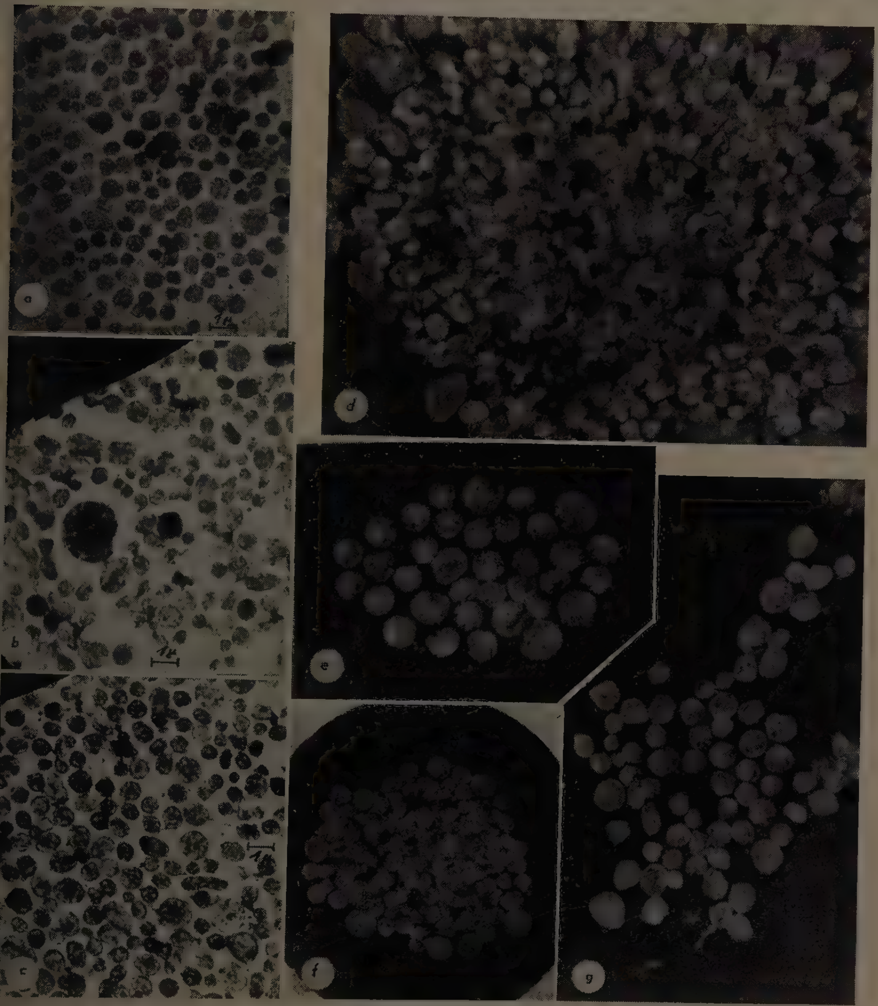


FIGURE 2. (*a* to *c*) Organisms of pleuropneumonia of cattle in correct position; agar xation on electron-microscope film; electron micrographs, unshadowed. (*d* to *g*) PPLO aidlaw-Elford strain A; microcolonies on electron-microscope film; agar filtration after Kel-nberger; electron micrographs, shadowed. The normal growth surrounding a microcolony ows no mycelialike structures.

Not much concerning the method of multiplication of the PPLO can be arned by these methods. More may be learned from continuous observation the single elements of a culture in the phase microscope, as I and my co-orker Zimmermann have done.[30] FIGURE 3*a* to *f* shows such a series of a aidlaw-Elford strain. Observation is difficult because of the small size of the organisms. Actually, the growth of the particles is seen better in reality than in the photograph. This growth probably takes place by a kind of sprouting, but not by forming long filaments and not through breaking up of the large forms, thereby liberating small units preformed inside the cells, as is often described as the regular method of multiplication.[26,31]

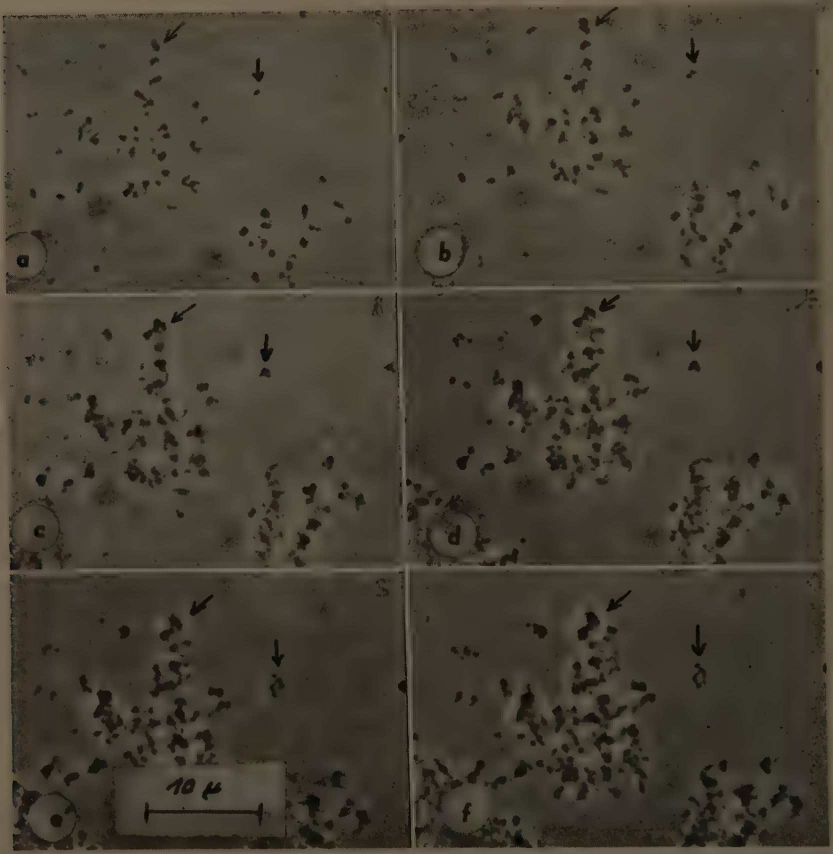


FIGURE 3. (*a* to *f*) Development of PPLO Laidlaw-Elford strain A on agar surface. The size of the cells lies on the limit of visibility. Phase micrographs.

Discussion

Examples of branching filaments and myceliumlike forms are often found in our material. These forms are sometimes stretched and pushed to one side. Under the phase microscope such "mycelia" can be produced merely by moving the micrometer screw because, by lifting or lowering the objective over the oil, a pressure variance is produced on the layer of fluid between the coverslip and the agar surface. It is essential for the interpretation of electron microscope pictures to perform direct optical microscope comparisons and to select the right method of preparation. Air drying of a suspension onto a film is unsatisfactory for electron microscopy.

Filamentous structures often are seen in the pleuropneumonia organisms of cattle and the agalactia organism and related strains, but very seldom in the sewage strains.[32] The latter are generally less soft and less fragile, and the colonies are less mucous than the colonies of the bovine strains.

According to other authors,[26,33,34] the filaments and mycelialike structures are not to be considered as growth forms of the PPLO. These forms also have no important or definite function in multiplication, because sprouting is not connected with a mycelium formation. Therefore, it is advisible to avoid such terms in classification or nomenclature. We cannot apply such terms to the less fragile PPLO strains, which almost never form filaments. Until now, no proof could be offered that multiplication occurs by the liberation of numerous granula through disintegration of a single large form. The elements, often called granula, are connected with a certain growth phase (FIGURE 4*f*) and are probably chondrioids. They play no role in the process of sprouting, contrary to some descriptions.[30,32,34,35] On this point I agree with Freundt.[36] It is necessary to distinguish between "accumulation of protoplasma" and "granula"; the latter are characterized by a stable configuration that is found also in the debris after breakup of a cell.

The presence of a cell membrane, as in bacteria, could not be shown morphologically; this is in accordance with comparative biochemical examinations of the cell wall of bacteria and PPLO.[37]

L FORM OF PROTEUS

Before discussing the morphology of the L form, it is necessary to define the concept. The suggested definition (SCHEME 2) deviates somewhat from the definition proposed by the Réunion sur les Formes L, Lausanne, Switzerland.[38] However the definition in SCHEME 2 appears usable for experimental work and conforms with the nomenclature of a series of recent publications.[39,40] We should refer to the L form only if the faculty to multiply as globular elements is present after change of the bacterial cell to the globular form. The border between the L form and the bacterial form is determined by the faculty of multiplication in form of the globular elements. It is not very important for the inception of the phenomenon whether this happens in the presence or absence of an inducing agent.

Morphology of the L form, type of colony, and type of multiplication become comprehensible through realization of the fact that morphologically different phases of growth are present in the population of the same culture.

The log phase in the case of the rod shape of *Proteus* is characterized by large cells rich in protoplasma and with demonstrable nucleoids (FIGURE 4*a*). Subsequent to this phase, small cells poor in protoplasma, with one or two chondrioids, are found (FIGURE 4*b*).

In the culture the globular form shows identical phases. The cells in FIGURE 4*c* and *g* are in the phase of potential multiplication; this phase is the same as the log phase of bacteria and may be called the log-phase equivalent. It is possible, by special methods, to obtain a population of cells nearly uniform in log-phase equivalent. The structure of the protoplasma corresponds to that of the rod form. Vacuoles and a cell wall are discernible. The latter is softer and more plastic than that of the bacterial form. After rupture, the remaining modified cell walls can be recognized (FIGURE 4*c*). During the log-phase equivalent, in the globular form as well as in the rod form, numerous nucleoids can be demonstrated by means of the optical and the electron microscopes (FIGURE 4*h* and *i*). The resting phase of the globular form shows a

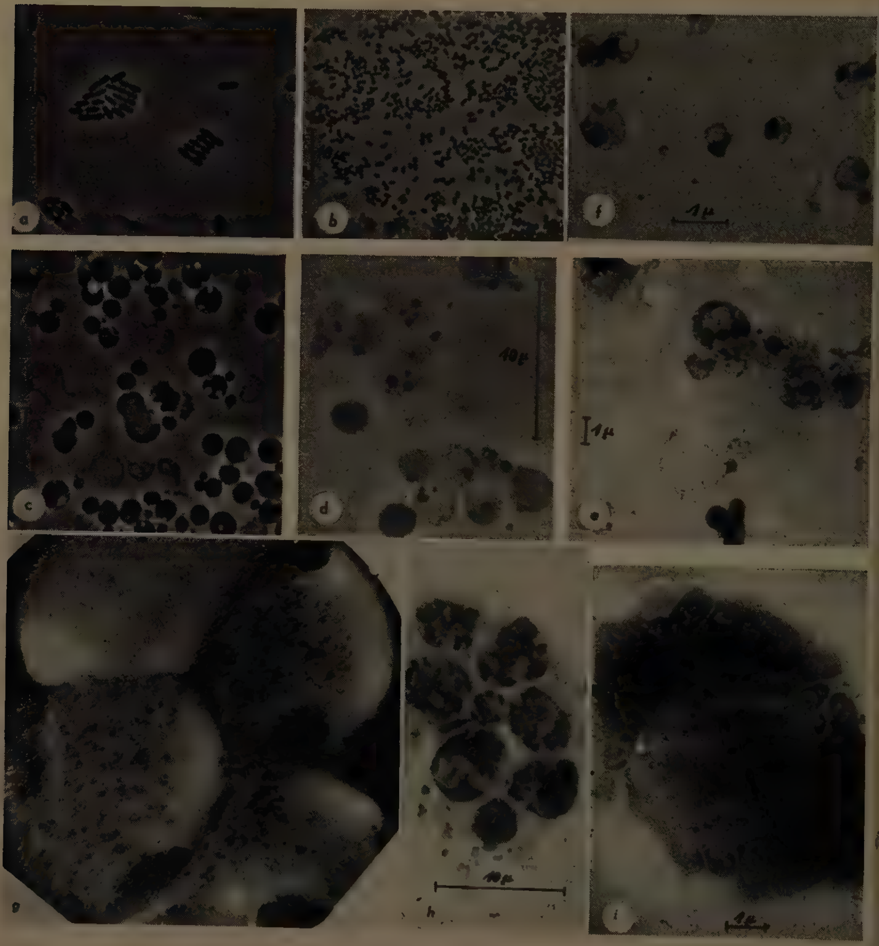
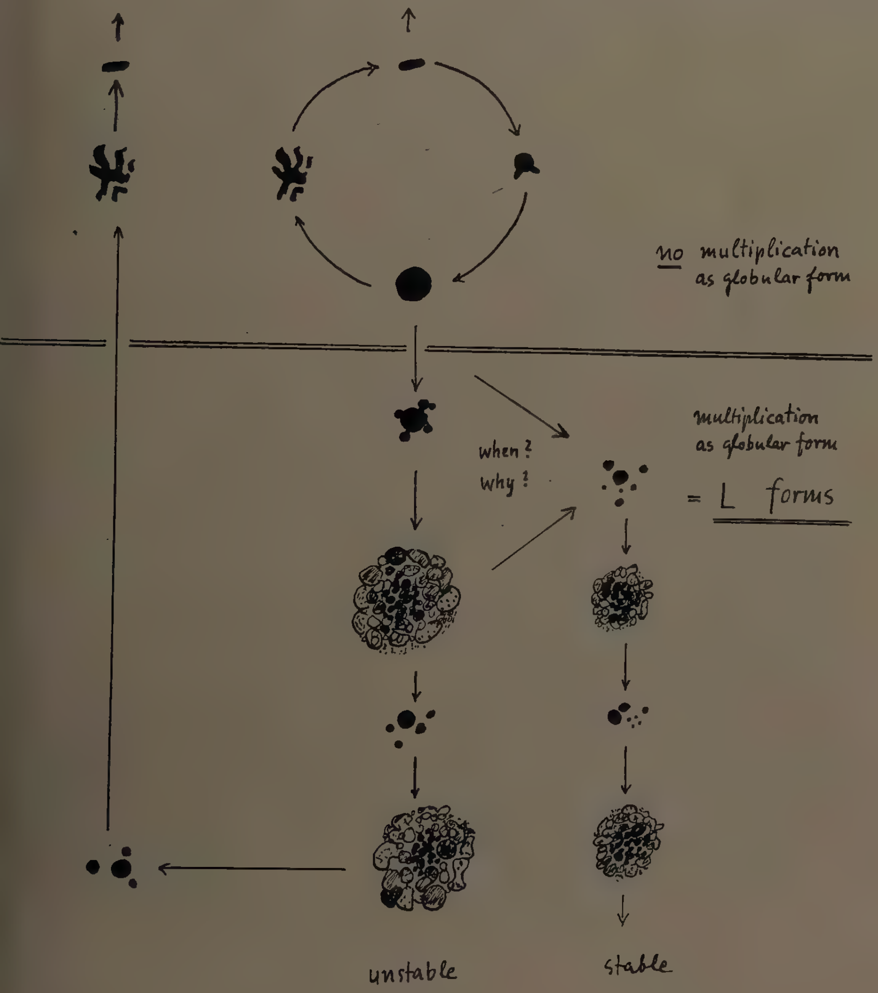


FIGURE 4. (*a*) Microcolonies of *Proteus* on agar surface. All cells are in the log phase. Phase micrograph. (*b*) Same strain under the same conditions, but cells are in the resting phase. In the light cells one or two dark granula are visible (chondrioids). Phase micrograph. (*c*) Globular forms of *Proteus* (same scale as *b*). Nearly all cells are in a uniform stage of growth. This phase is identical with the phase of the bacterial form of *Proteus* in *a* and should be called log-phase equivalent. Some cells are lysed and the cell membranes remain in the liquid. Phase micrograph. (*d* and *e*) The same objective as in *c*, but nearly all cells are in the resting phase (corresponding to the bacterial form in *b*). In every cell some chondrioids are visible. (*d*) Phase micrograph. (*e*) Electron micrograph. (*f*) PPLO Laidlaw-Elford strain A. The same stage of growth as cells in *b*, *d*, and *e*. Chondrioids appear inside the cells. Electron micrograph. (*g* to *i*) Globular forms of *Proteus* in the log-phase equivalent. (*g*) Dense cells, some vacuoles in the protoplasma. (*h* and *i*) In the log-phase equivalent, numerous bacterial nuclei are demonstrable for every cell. (*h*) Robinow's method, ordinary micrograph. (*i*) Kellenberger's method, electron micrograph.

morphology corresponding to the resting phase of the rod form: that is, transparent cells, poor in protoplasma and containing some intracellular granula (FIGURE 4*d* and *e*). The same phenomenon is also found in the PPLO (FIGURE 4*f*). To demonstrate the actual process of multiplication in the L form


no multiplication as globular form
when? why?
multiplication as globular form
= L forms
unstable
stable

SCHEME 2.

s more complicated than in the case of the bacterial form. The process takes lace only in a small part of the unstable globular form on the agar surface. selective growth of colonies is also found in a chance furrow on the agar urface despite uniform superficial inoculation. If globular elements are mbedded in the agar layer, the rate of multiplication is constant, between 5 15 per cent. The unstable type of the L phase is particularly suited for uch studies. By the capacity of the unstable L phase to revert to the bacterial form, the amount of colony-forming units can be determined quantitatively. In a few isolated elements, which lie near the surface, the first steps of multiplication may be observed. FIGURES 5 and 6 show multiple, simultaneously starting sproutings; mother cell and daughter cell have identical structures. The multiplication of PPLO may be visualized in a similar way.

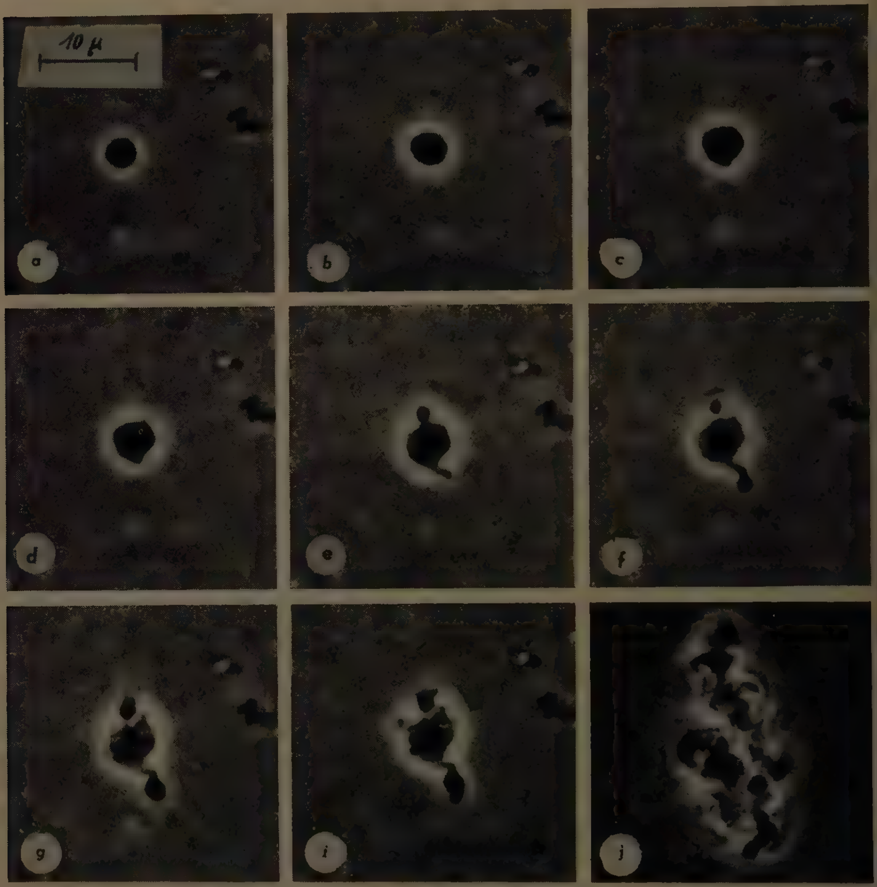


FIGURE 5. (*a* to *j*) Development of a globular form of *Proteus* to a microcolony. The cell lies *in* the agar a short distance under the agar surface; at the same time, sprouting is starting at two points (*d* to *f*). The beginning of two additional sproutings is visible in *i*. The tridimensional growth of the microcolonies does not admit further observation of details. Phase micrographs.

The globular form of *Proteus* on the agar surface is unable to multiply in most cases. FIGURE 7*a* to *p* shows the development from the rod form to the globular form and the behavior of the globular cell. During a great part of the growth period the globular form extends pseudopodialike protrusions into the surroundings. During this period the margin of the cell fluctuates constantly, but there is no separation of the protrusions. Finally, the cell obtains an even margin and vacuoles appear and disappear in different places in the protoplasma. The end of the phase of potential multiplication is reached, and the resting phase begins. The morphology of this phase is characterized by transparent cells, poor in protoplasma and rich in granula (FIGURE 7*p*; compare with FIGURE 4*b*, *d* and *e*).

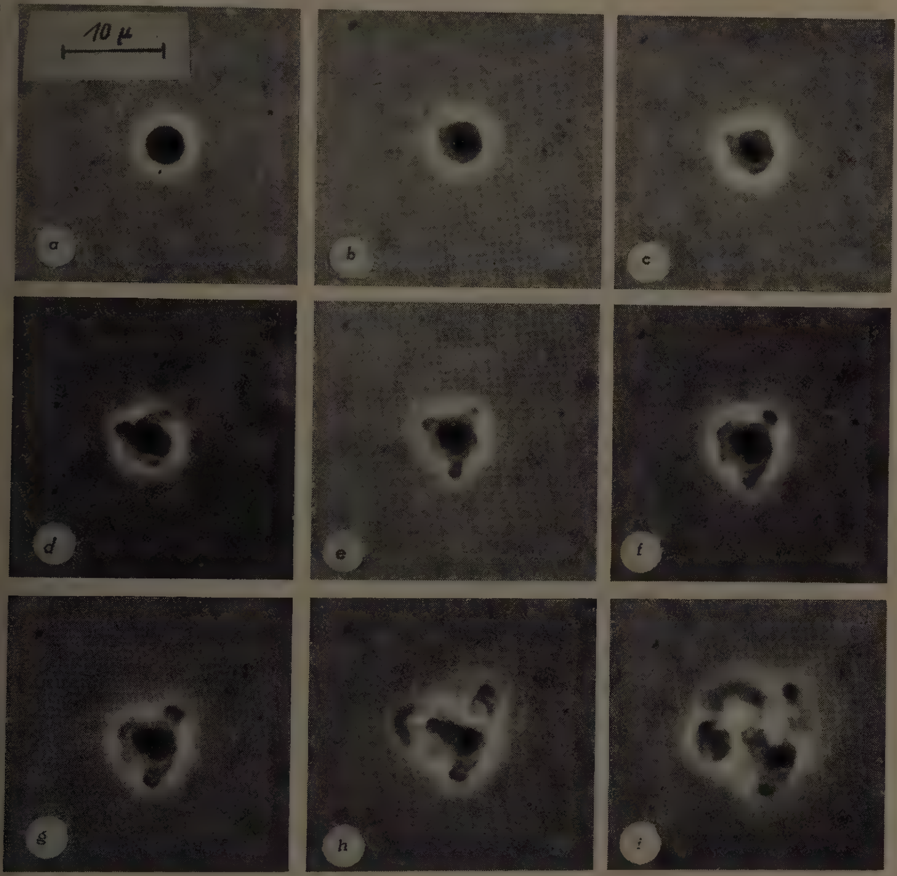


FIGURE 6. (*a* to *i*) Another cell showing a procedure similar to that in FIGURE 5.

The shallow depth of vision of the optical microscope prevents continuous observation of the tridimensional growth of a microcolony in the agar layer. Only when the L colony breaks through the agar surface and spreads does exact observation become possible. Surprisingly enough, there is no multiplication in the marginal zone of the colonies as in the bacterial form (FIGURE 8*a* to *l*). All young cells of the globular form appear from the depth in the center of the colony, and the change of position in the outer zone is only of a passive nature. At the end of the observation, as in the beginning, the same cells of the globular form are found in the margin (FIGURE 9*a* to *f*). In aging, their structure has changed typically. Some of them, after complete breakup, are present only in debris. Throughout the conglomeration of cells in different growth phases and in the debris new cells are observed in the stage of the log-phase equivalent. In this way the perplexing picture of an L colony is produced (FIGURE 9*g*): toward the outer border lie cells of the globular form in different phases of development. They are partly deformed, of different protoplasma content, often vacuolized, and often contain numerous granula (chondrioids). Foamy structures and extracellular granula are visible between the cells. These are remainders of disintegrated globular bodies (the rise of young cells from the center has been demonstrated also in a time-lapse film by Udo Taubeneck of Jena, Germany).

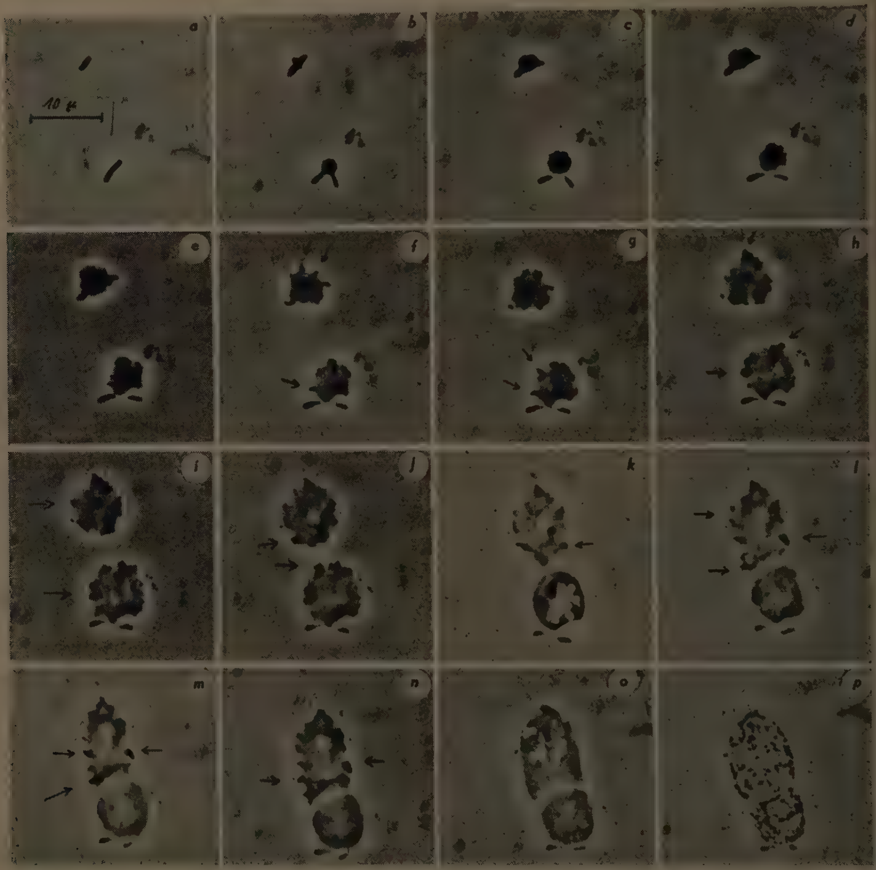


FIGURE 7. (*a* to *p*) Development of two rod-form cells of *Proteus* to the globular form. In contrast to FIGURES 5 and 6, the cells lie *on* the agar surface and attempts to multiply are fruitless. Note that the regular cell division of the lower cell is imminent if the breakup to the globular form occurs. Arrows show some of the attempts at sprouting. Attempts are seen to be abandoned by one cell in *k* and by the other in *o*. After leaving the log-phase equivalent, both cells enter the resting phase, characterized by numerous granula and chondrioids (*p*). Phase micrographs.

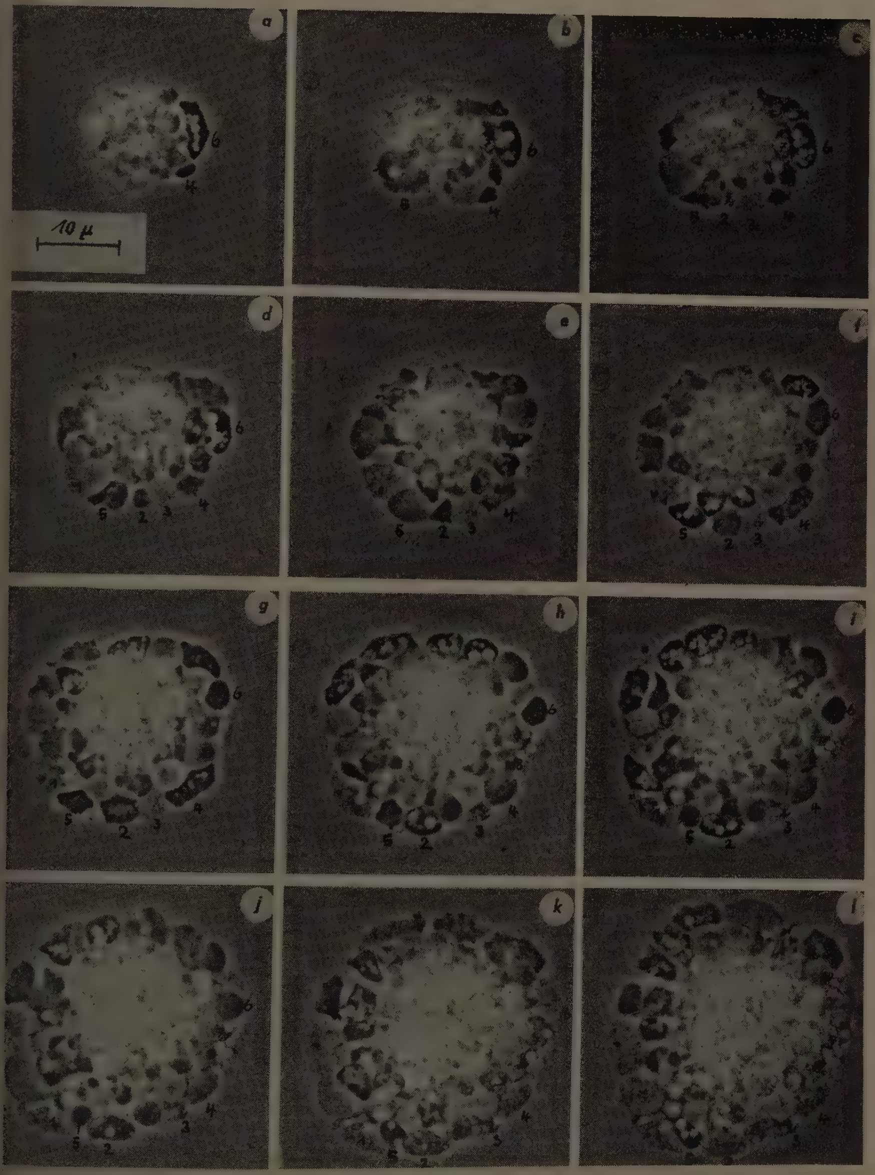


FIGURE 8. (*a* to *l*) Development of an L-form colony (Type 3B) on the agar surface as the subsequent process of FIGURES 5 and 6. Multiplication happens only in the depth of the center of the colony. The margin zone of the colony shows elements without processes of multiplication. Lysis and transfer to the resting phase can be observed on single cells. Phase micrographs.

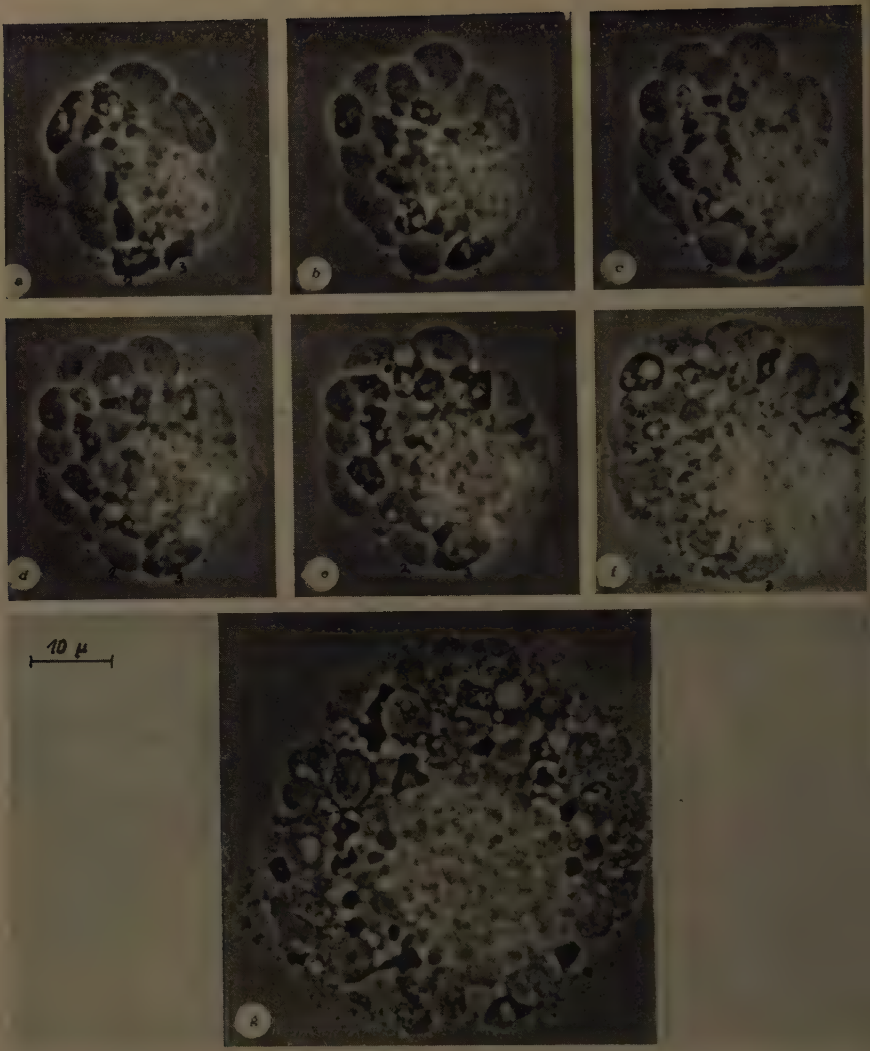


FIGURE 9. (*a* to *g*) Another example of the development of an L-form colony. In the margin zone the L colony shows (*g*) a perplexing jumble of cells in different growth stages (generally in the resting phase), of debris after lysis, and of some elements in the log-phase equivalent. The log-phase equivalent cells are ejected from the depth of the center. Phase micrographs.

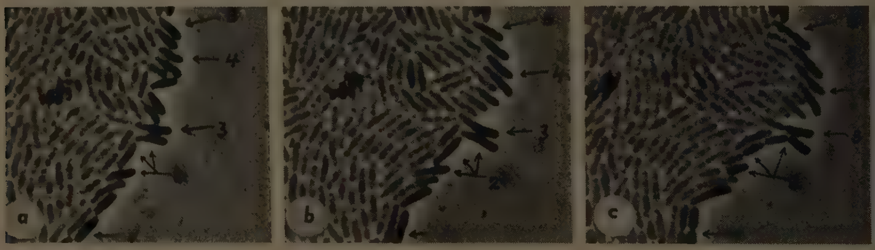


FIGURE 10. (*a* to *c*) The margin zone of a growing colony of *Proteus*. All cells are in the log phase. The cells of the far margin also show steps of multiplication by division.

The rod form of bacteria also multiplies in the marginal zone of the colony. These colonies do not show differences in the phases of development of their population (FIGURE 10). An accumulation of elements that cannot multiply in the outer part is typical for the L colonies. The reversion of a single globular element of *Proteus* to rod-form cells is shown in FIGURE 11*a* to *l*.

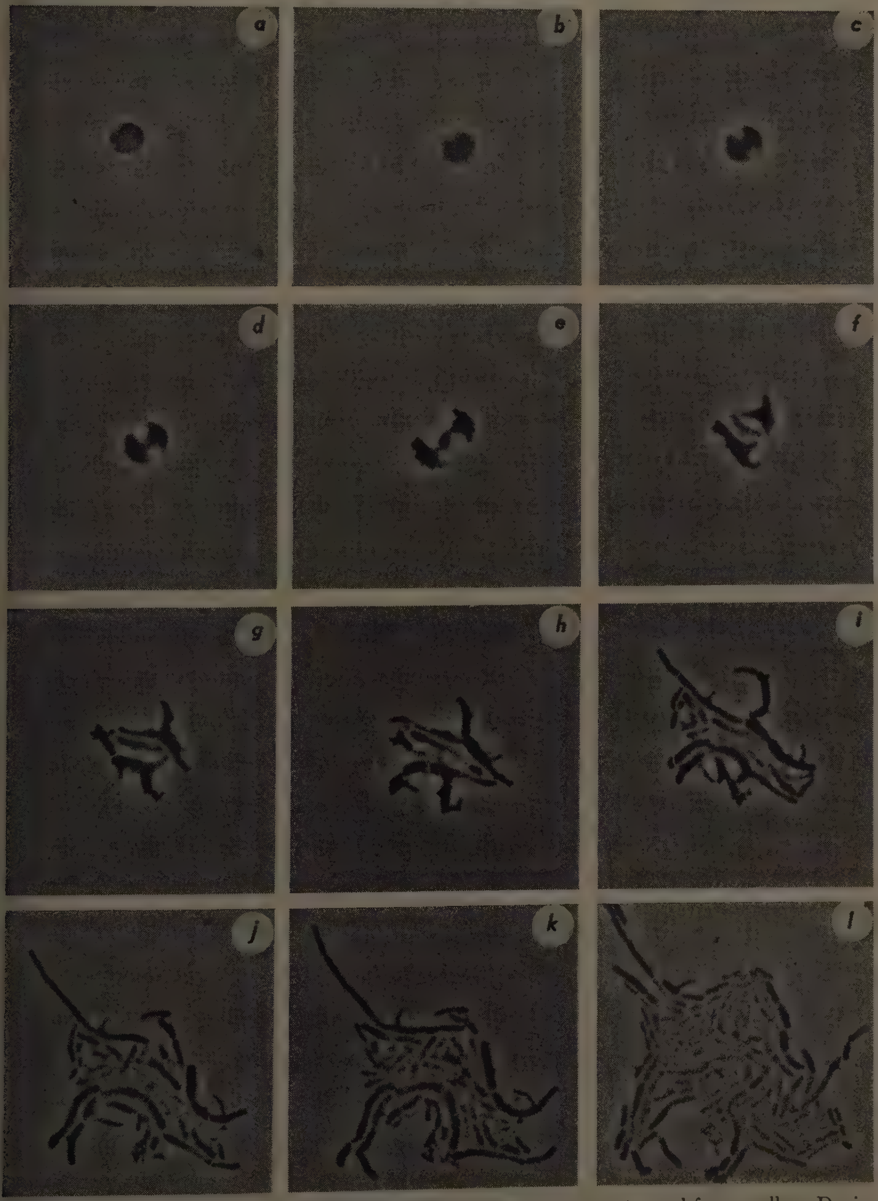


FIGURE 11. (*a* to *l*) A globular element of *Proteus* returns to rod-form cells. During the transformation, branching elements are found.

FIGURE 12*a* to *d* shows microcolonies of the stable L form of *S. moniliformis.* The elements differ considerably in size. Small globular elements, as well as large bodies of great plasticity, are found. The cell wall cannot be compared with that of the bacterial form and is also of a different nature than that of the unstable L form. Particular structural forms of the smallest elements cannot be seen; apparently, mother cell and daughter cell are structurally identical. Those strains of the stable L form that I examined are morphologically comparable to the PPLO (FIGURE 2*d*), but compared to the PPLO they have more irregular dimensions.

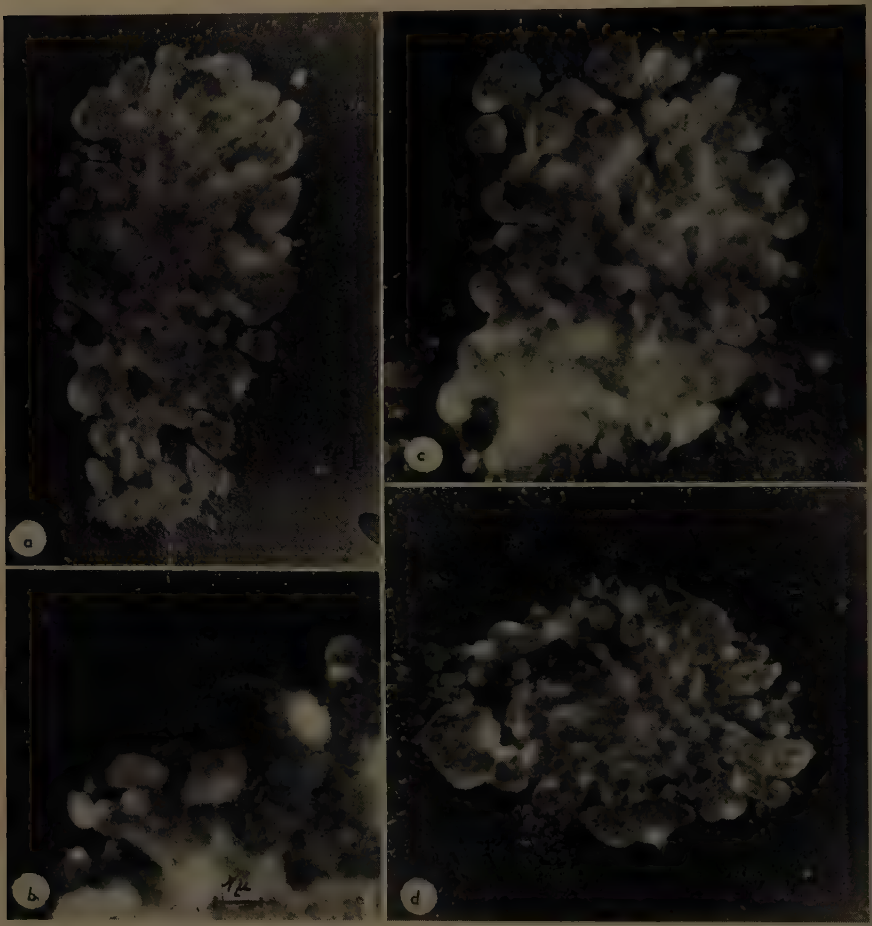


FIGURE 12. (*a* to *d*) Microcolonies of stable L form (*Streptobacillus moniliformis*). The elements are morphologically identical to those of PPLO in FIGURE 2, but somewhat more variance in size can be observed. Lighter and larger elements are bordered by smaller and denser ones. In the stable L-form colony, as well as in the unstable L-form colony and in the PPLO colony, some intermingling of cells of different growth phases occurs. Agar filtration after Kellenberger. Electron micrographs.

Discussion

The results of the morphologic examination of the L form coincided with results obtained by other methods such as serial filtration combined with counting of viable elements,[41] biochemical analysis,[37,42] and phage adsorption.[40,43] It was proved conclusively that the cell hydrolyzate of the stable L-form type, in contrast to the nonstable type, contains no basic structure[37] and that phage adsorption does not take place.[40,43] The special structure of the cell surface appears to be connected with the fact that the smallest elements of the stable type are filterable, like the PPLO, and that the elements of the nonstable type are not filterable. In the case of the stable L form and of the PPLO, the filterable elements differ from the nonfilterable only by their size and not by their structure.[41] The development of the L form is not a result of the fusion of two rod cells[44] or of the fusion of granula that are freed from transparent rod cells.[9,45,46] The first globular element arises by a sudden breakup of a bacterial cell and by spreading out through the crack[47,48] (as demonstrated by a time-lapse film[49]).

The protoplasts are to be considered, to a certain degree, as a phenomenon similar to the L form. However, we cannot assume, for different reasons, that they are equivalent to the L form.[40,50] An essential difference lies in the fact that the protoplast cannot grow or multiply, whereas the concept of the L form includes this possibility.

In the L form and the PPLO the granula often were considered as independent units of multiplication, set free by disintegration of the mother cell. Some authors regard the granula as the morphologic substrate of the filterable elements that were capable of multiplication.[9,26,45,46,51-53] Not only the direct morphologic observation, but also the quantitative determination of the units of multiplication before and after filtration[41,46] cause us to regard the granula as chondrioids. The complicated theories about multiplication appear to be false; there is no reason to assume that there exists a cycle of multiplication through breakup of large bodies into numerous granula. The processes of multiplication of the L form and the PPLO are simple and of the same kind. The regular multiplication takes place through some kind of sprouting. In the case of the L form, the process of sprouting is dependent upon the surroundings and is not regular under all conditions.

The examinations of the L form are complicated, because the cells of the population of a growing culture are in different phases of development. Our investigations are facilitated essentially by fulfilling those conditions that produce a cytologically homogeneous material of globular elements.[47] The reason that the development and multiplication of the L form remained obscure for a long time was the intermingling of various stages of development in one colony, as well as the accumulation of cells that were unable to multiply, in the marginal zone.

The stable L form and the PPLO are equal morphologically; however, it is necessary to keep them separate until our knowledge permits a final decision.

SUMMARY

PPLO and the L form have a similar morphology. In both cases multiplication takes place by a sprouting process, and parallels to the growth phases

of bacteria are to be found. There are differences in colony growth between bacteria and the L form.

ACKNOWLEDGMENT

The micrographs in FIGURE 1*g*, FIGURE 2*d* to *g*, FIGURE 4*a* to *e* and *g* to *i*, and FIGURE 12*a* to *d* are derived from papers written with E. Kellenberger; they were produced in the Laboratoire de Biophysique of the University of Geneva, Geneva, Switzerland.

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THE CELL WALL OF BACTERIAL L FORMS AND PLEUROPNEUMONIALIKE ORGANISMS*

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Plasticity and fragility are important characteristics of the pleuropneumonialike organisms (PPLO) and L forms of bacteria. Mechanical fragility may be demonstrated easily by observing rupture of the large round organisms when slight pressure is applied to the coverslip of a slide preparation, and osmotic fragility is demonstrable by examination of suspensions of the organisms in solutions of varied osmotic strengths. It should be emphasized that these observations refer to structural integrity and do not relate necessarily to viability, an interesting dichotomy recently noted by Smith.[1] Nevertheless, the degree of pliability and fragility exhibited by these organisms reflects one of the most striking differences that is observed when this group is compared with bacteria. The property of rigidity in bacteria is known to be dependent on the bacterial cell wall. Thus the cell wall, isolated from the bacterial cytoplasm following rupture of the cell, retains the shape of the original bacterium.[2,3] Conversely, lysozyme-sensitive bacteria become spherical in shape when treated with this enzyme in an osmotically protected environment,[4] and this type of treatment is known to dissolve the isolated cell walls of the same bacteria.[5] This evidence is sufficient to conclude that the PPLO and L forms do not possess a cell wall with the same structural properties as the bacteria. Of course, this is not a new conclusion. Such a description as that of Nowak, who said that "our corpuscles seem to be droplets of a thick liquid protoplasm not yet consolidated and stabilized," attests to the early recognition of this property.[6] With reference to the bacterial L forms, Dienes and Weinberger stated that "it is apparent that *Proteus* can survive devoid of the usual bacterial membrane, and that the L form seems to be the growth of the elementary units of bacteria without a membrane."[7]

It is perhaps appropriate to ask first whether the PPLO and L forms are completely devoid of a membrane or whether they merely lack the "usual bacterial membrane." Morphologic studies on this point would appear to be conclusive. Thus in most instances a membrane can be observed in stained preparations, in unstained preparations examined with the phase microscope, or in sectioned material examined in the electron microscope. This has been demonstrated clearly by Thorsson and Weibull[8] and by van den Hooff and Hijmans,[9] and is illustrated here by the author's micrographs of a streptococcal L form, a PPLO, and a streptococcus (FIGURE 1).

If one accepts these observations as sufficient proof of the existence of a membrane, the next question is whether the membrane in the L form is the cytoplasmic membrane of the original bacterium or an altered cell wall. The

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first evidence regarding this question was obtained in the course of studies on immunological differences between the bacterial and L forms of streptococci.[10] In those studies it was found that the group-specific polysaccharide was lacking in the L form. Previous immunological studies on L forms of other species

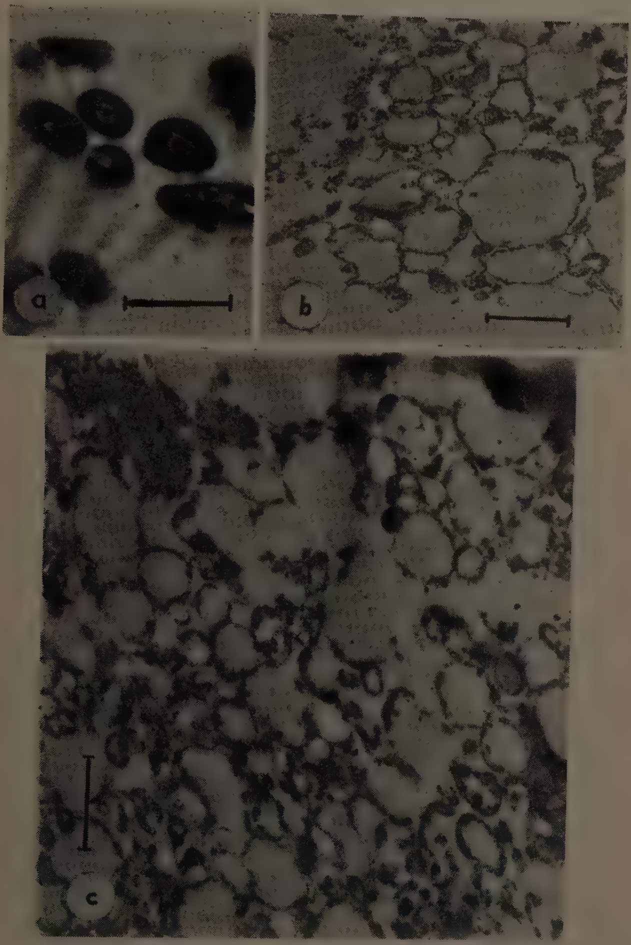

Figure 1. Electron micrographs of sectioned bacterium, L form, and pleuropneumonia-
ke organism. (*a*) *Streptococcus hemolyticus*, ×13,960; (*b*) L form of *Streptococcus hemolyti-*
us, ×11,480; and (*c*) K5 goat strain PPLO, ×12,250. The PPLO strain was furnished by
enry E. Adler.

ad demonstrated in several instances that the L forms lacked antigenic com-
onents present in the bacterial form (for example, *Streptobacillus*[11] and *Pro-*
us[12]). These earlier studies had not established an alteration in any structural
omponent of the cell, nor in fact were they intended to do so, since they ante-
ated the immunological and chemical characterization of isolated bacterial
ructures. Two factors made it possible to interpret the studies on strepto-

cocci as reflecting structural modification. First, a single antigenic constituent was studied rather than the total antigenic complex. Second, McCarty's studies had demonstrated the localization of the group-specific polysaccharide in the cell wall of the bacterium,[3] making it possible to conclude that the L form of the streptococcus had a markedly altered membrane structure. However, it was not possible to distinguish between the total loss of the cell wall and the loss of a portion or even a single constituent of the wall.

In order to determine whether the absence of the polysaccharide in the L form of the streptococcus represented failure of synthesis, studies were conducted to determine whether the polysaccharide was excreted into the fluid medium or whether it was metabolized. Experiments relating to the first of these questions are illustrated in TABLE 1. The results indicate that the supernatants of broth cultures of the L form may be concentrated from 200 to 500 times and still give negative serologic reactions for the group-specific polysaccharide. It was demonstrated next that the group-specific polysaccharide was not metabolized by the L form, even after five serial subcultures in the presence of the carbohydrate. This is illustrated in TABLE 2. From these studies it was concluded that the L form was incapable of making the cell wall polysaccharide.

TABLE 1

Material	Concentration	Method of purification*	Precipitin reactions with Group A antisera
AED-L culture supernatant	520:1	zinc-ethanol	negative
D58-L culture supernatant	200:1	zinc-ethanol	negative
AED-L culture supernatant	200:1	chloroform-ethanol	negative
ADA-L culture supernatant	200:1	chloroform-ethanol	negative
Broth with Group A substance	100:1	zinc-ethanol	positive
Broth with Group A substance	200:1	chloroform-ethanol	positive
Broth—no additions	100:1	zinc-ethanol	negative
Broth—no additions	200:1	chloroform-ethanol	negative

* Zinc-ethanol method: ethanol and zinc acetate added to final concentrations of 19 per cent and 0.02 M. Precipitate discarded. pH raised to 7.5. Precipitate discarded. Residual zinc removed with Dowex 50 (H). Dried on vacuum still.

Chloroform-ethanol method: ethanol added to final concentration of 50 per cent. Precipitate discarded, supernatant dialyzed and concentrated to small volume. Shaken with chloroform. Precipitate discarded. Four volumes of 95 per cent ethanol added. Precipitate discarded. Dried in streaming air.

The question was then raised as to whether the absence of important cell wall constituents was a general property of all L forms. Our attention was turned to a strain of *Proteus* and its L form.* Since these studies[13] are to be reported in detail elsewhere, they will be summarized only briefly here. A bacterial antigen with many of the characteristics of a somatic antigen, containing a relatively high concentration of amino sugar and reducing substance, was found also in the L form. Studies on separated cell walls demonstrated a high concentration of this antigen in that structure of the bacterium. However, the antigen was found also in sizable amounts in the bacterial cytoplasm, and it could not be determined with certainty that this antigen was present in the cytoplasm only as a result of incomplete separation from cell wall constituents. Thus the presence of the carbohydrate-containing antigen in the L form does not indicate conclusively the persistence of a bacterial cell wall constituent in the variant form, although this is a reasonable explanation of the observation.

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* *Proteus* 18 and its L form were kindly supplied by R. Tulasne, Strasbourg, France.

At this point it is perhaps worthwhile to outline some of the possible alterations in the bacterial membrane structure that might result from the transformation of a bacterium to an L form and be associated with the morphologic alterations that are observed. In the electron micrograph of a sectioned bacterium (FIGURE 1) it may be seen that the cell wall is a relatively dense structure of approximately 150 Å thickness and with the double osmiophilic density pattern characteristic of many membranes. Little else indicates the nature of the outer bacterial membranes. Although chemical complexity of bacterial cell walls has been established,[14] the manner in which the proteins, polysaccharides, and lipids are organized into a rigid structure is as yet unknown.

TABLE 2

POSITIVE REACTIONS OBTAINED WITH GROUP A ANTISERA

Broth supernatant	Minimum concentration* necessary for positive reaction
Uninoculated broth, Lot No. 3, Group A substance†	2.5:1
XIII*b* AED-L_{117} cultured in broth, Lot No. 3, with Group A substance	2.5:1
XIII*c* AED-L_{118} cultured in broth, Lot No. 3, with Group A substance diluted 1:10	25:1
XIII*d* AED-L_{119} cultured in broth, Lot No. 3, with Group A substance diluted 1:10	25:1
XIII*e* AED-L_{120} cultured in broth, Lot No. 3, with Group A substance diluted 1:20	50:1

* Broth supernatants purified and concentrated to final volume by zinc-ethanol method outlined in TABLE 1.

† Bacterial organisms extracted with hot acid, neutralized, added to uninoculated broth so that extract from 1 ml. growth of organisms was included in 1 ml. of final broth mixture; *b*, *c*, *d*, and *e* are second, third, fourth, and fifth serial subcultures on broth containing group-specific polysaccharide.

In any case, it would appear that the loss of rigidity that accompanies transformation of a bacterium to its L form could result from a number of either simple or complex alterations. At one extreme, it is possible that the L form is contained by the bacterial cytoplasmic membrane as a result of the complete loss of the bacterial cell wall. At the other, it is perhaps conceivable that no loss of wall constituents has occurred, but that an altered organization of components—for example, a decrease or absence of cross linking—is responsible for the altered morphology. Between these two extremes the loss of certain specific components among the wide variety of chemical constituents of cell walls that have been described conceivably might result in the observed effect. It is unnecessary to postulate that the alteration is uniform from one species of L form to another or, for that matter, from one line to another within the same species. It is only necessary to recall the marked morphologic differences between the 3A- and 3B-type colonies derived from single *Proteus* strains to emphasize the importance of this consideration.[15] Nevertheless, it is important to consider the possibility that a component of the wall common to many organisms and necessary to rigidity of the bacterial form is absent from all L forms, variations from one L form to another being due to superimposed changes. What might such a component be? Although little is known about the macromolecular organization of the bacterial cell wall, great strides have been made in recent years in characterizing some of the smaller components. It has been especially striking that a recently described distinctive amino sugar, muramic acid, and an amino acid, diaminopimelic acid, in combination with alanine and glutamic acid have been observed to occur together in the bacterial cell walls of a number of species with such regularity as to be considered a basic cell wall component.[16,17] In addition, it has been established that muramic acid is the amino sugar in the uridine-diphosphate nucleotides described by Park.[18] These nucleotides were demonstrated earlier to accumulate in a *Staphylococcus aureus* treated with penicillin during the active growth phase.[19] Since it has been proposed that penicillin acts by blocking the incorporation of this peptide-amino sugar component into the cell wall of the bacterium,[18] it would follow that this component should be lacking in the L form if two conditions are fulfilled. First, it must be reasonably certain that the altered morphology of the L form is a result of penicillin effect and, second, it must be demonstrated that a pathway for the incorporation of the peptide-amino sugar component independent of penicillin action has not developed.

Do these criteria apply? There is some reason to believe that they do. Exposure of sensitive bacteria to penicillin is followed by a remarkably uniform set of events. Within a few hours, or at most a day or two, the bacterial cells become enlarged and swollen. A variable proportion of these altered cells is capable of continuing growth and reproduction in this form, and these are, of course, the L forms of bacteria. The first stage in this series of alterations should be construed in no way as a specific effect of penicillin, since the swelling of bacteria to large bodies, or protoplastlike structures, has been observed to occur spontaneously and following exposure to a wide variety of noxious agents. To list only a few of the important examples, many antibiotics, toxic cations, appropriate concentrations of antibody and complement, and at least two amino acids repeatedly have been observed to produce the swelling of bacteria. In some of these examples production of L colonies from the altered bacteria has been observed, but in most instances the altered forms have been incapable of continued growth. Although the small size of the organisms has precluded any extensive morphologic study of subcellular structures, the observation of growth in the altered form induced by penicillin would seem to indicate that in this instance the alteration in form might represent a more specific effect of the antibiotic than in those instances where the rounding and swelling up of the bacterium appears to represent a stage in cell death. The fact that L forms from many species become "fixed" or "stable" strains that do not revert to the bacterial form on withdrawal of penicillin does not exclude the possibility that the morphologic alteration is a specific effect of the drug, since it is conceivable that during subculture in the L form the organism loses the cytoplasmic capacity for synthesis of the cell wall or a cell-wall component. This could be the result of an alteration in genetic material,[20] but it should be appreciated that there are examples of transmissible cytoplasmic alteration without genetic change.[21,22] Further evidence for the specificity of penicillin effect is provided by the observation that with this drug L forms have been induced to grow from many bacterial species that have never been observed to produce L forms spontaneously. In addition, it has been observed that L forms that have arisen spontaneously or that have been obtained by procedures other than exposure to penicillin are fully as resistant to that antibiotic as are strains obtained by exposure to the drug. This observation is taken to indicate that the organism that lacks the drug target, namely the cytoplasmic unit responsible for wall synthesis, is not affected by the drug. The sum of these considerations suggests that the altered morphology of the L forms is a direct result of penicillin effect when that drug is used for their production. It is, of course, possible that the alteration in the cell wall is one among multiple changes with or without a factor common to all. Because of the small size of the bacterial structures, other alterations almost certainly would have missed our recognition, but there is no evidence for other fundamental structural changes, and this possibility will be dismissed without further consideration here.

The second point is also important, namely the demonstration of the absence of an alternative pathway for cell wall synthesis. This point is not always easy to establish, as may be illustrated by some observations of Ward on a *Proteus* bacterium recovered from an L form.[23] When this organism was inoculated on penicillin-containing plates, it produced colonies composed entirely of large bodies. Initially, these were believed to be L colonies despite a few atypical features. However, when the organism was grown in liquid it became clear that it was penicillin-resistant in the bacterial form. Thus, when inoculated into deep tubes, most or all of the cells were converted to large swollen forms, and the density of growth was poor unless salt or sucrose was added to the medium. These results were the same whether penicillin was included in the broth or not. Furthermore, when the organism was grown in a shallow layer of broth aerated by constant rotation on a shaking machine, dense growth consisting almost entirely of bacterial rods was obtained. Again, the results were the same whether or not penicillin was included in the medium. When Ward went back to the line propagated on penicillin plates that grew as colonies of large swollen forms, he observed that this strain also grew as a bacterium in penicillin broth when aerated vigorously. Further studies have not been performed on this variant, and these observations have not been reproducible at will, so their occurrence may be of little significance. Nevertheless, these observations are presented to emphasize that not all morphologic variants of bacteria, even when those variants take a large swollen shape, are typical L forms. In addition, it illustrates that morphologic variation occurring in the presence of an antibiotic is not necessarily an effect of that antibiotic.

The reports of Kandler *et al.*[24] and of Weibull[25] on the examination of L forms of bacteria for diaminopimelic acid (DAP) and muramic acid merit consideration at this point. Kandler and his associate chose to examine only a fraction of the bacteria or L forms. This fraction was the insoluble residue which remained after incubation of the organisms with 1 N NaOH at 60° C. for 2 hours followed by further incubation in 1 N NaOH for 12 hours at 37° C. These investigators reported studies on a stable and an unstable L form, describing the biological properties of these strains only by stating that the stable form did not return to the bacterium, whereas the unstable form did. These studies demonstrated muramic acid and DAP in the unstable L form. Because they found no alkali-insoluble residue in the stable L form, these researchers concluded that this strain lacked completely the basic cell-wall structure.

Weibull took a somewhat different approach in studies conducted on a *Proteus* L which, he stated, showed no tendency to revert to the bacterial form. He extracted the organisms with trichloroacetic acid (TCA) and ether and examined the insoluble residue for content of hexose, hexosamine, and DAP. His results showed considerable reduction in these components in several batches of the same L form grown on different media and, in one instance, he was unable to recover DAP from the L form, using techniques estimated to be sensitive for approximately one seventh the amount found in the whole bacterium.

In view of the importance of this issue, I have conducted studies characterizing the hexosamines of the bacterial and L forms of a *Proteus* strain. It has been deduced that the membrane of the L form is either the cytoplasmic membrane of the bacterium or an altered cell wall, and it is possible that the morphologic alteration might result in altered solubility properties. For this reason the studies reported here have been conducted on the insoluble residue following TCA extraction. The bacterium and L form of the *Proteus* strain* have been maintained by continuous subculture. The L form has not been observed to revert to the bacterial form, either spontaneously or in any of the attempts to induce reversion. It grows predominantly as large swollen forms when cultivated in liquid and frequently forms a spreading film at the surface of broth cultures.[26] The bacteria, grown in broth, were collected by centrifugation, washed with saline solution, and then suspended in distilled water for dialysis in the cold against several changes of water. Some lots of the L form were handled in the same way, the organisms being extracted with TCA after dialysis. Other lots of the L form were extracted with TCA immediately after collection from broth. Residual TCA was removed by washing with ether. Thereafter the bacteria and L forms were handled in the same way. Weighed portions of the organisms or TCA residue, dried by lyophilizing, were hydrolyzed in sealed tubes with 6 N HCl in a boiling water bath for 6 hours. The HCl was removed by evaporation, and the hydrolyzate was applied to Dowex 50 (H) columns for chromatography, using the system outlined by Gardell.[27] Aliquots of every second or third tube were used for amino sugar determination, using a modified Elson and Morgan procedure,[28] and the remaining tubes were used for identification procedures. FIGURE 2 is the elution diagram from the chromatography of the hydrolyzate of 250 mg. of bacteria. The hexosamine values are plotted against milliliters of effluent, revealing five peaks that are designated by capital letters. Peak A has a slow R_f on paper chromatography relative to known hexosamines and is presumed to be incompletely hydrolyzed material without further identification. Peak B has been identified as either glucosamine or mannosamine using the Stoffyn-Jeanloz Ninhydrin degradation technique.[29] The material is presumed to be glucosamine, but it should be appreciated that neither the column chromatography nor the paper chromatography used here for separation and identification will distinguish between these two amino sugars. Similarly, Peak D has been identified as either galactoseamine or talosamine. Identification of Peak E has not been attempted, but it is noteworthy that this material is eluted from the column at approximately the same place as fucosamine[30] and as the recently described amino sugar component of a *Bacillus subtilis* polysaccharide reported by Sharon and Jeanloz.[31] Peak C is incompletely separated from both B and D, and consequently is a mixture of those two amino sugars plus a third compound. The latter was identified as muramic acid, using the following procedure. The tubes indicated within brackets on the diagram were pooled, HCl was completely removed by evaporation, and the amino sugars were dissolved in water and applied to columns of Amberlite IR-120 buffered[32] at *p*H 6.05. In this procedure glucosamine and galactosamine are absorbed to the resin, and muramic acid appears in the eluate. An Elson-Morgan reaction was performed on an

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* *Proteus* 18.

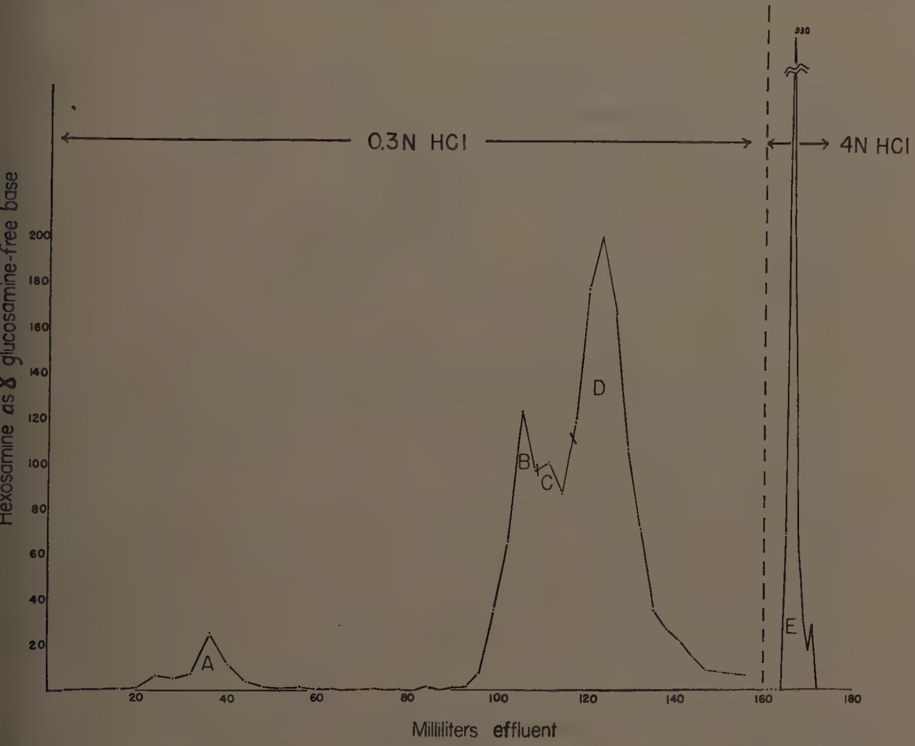

FIGURE 2. Hexosamine chromatogram IX (Dowex 50, 1 × 33 cm.). Hydrolyzate of *Proteus* 18, 250 mg.

aliquot of the eluate from the Amberlite column to quantitate the muramic acid, and final identification was obtained by paper chromatography.* FIGURE 3 is a reproduction of paper chromatograms showing the identification of the peaks in this column separation.

The next elution diagram, FIGURE 4, was obtained from the hydrolyzate of 0.892 gm. of acid-extracted *Proteus* 18 L residue. Peaks comparable to A, B, D, and E of the bacterial diagram are recognizable; B and D were identified as glucosamine (or mannosamine) and galactosamine (or talosamine). No peak comparable to C of the bacterial elution diagram is present in the experiment using the L form. To be certain that muramic acid was not present in small amounts and buried in the B peak or the trough between the B and D peaks, the tubes in the area indicated on the diagram were pooled, dried, and passed over an Amberlite column. Muramic acid could not be identified in the eluate. Furthermore, an Elson-Morgan reaction performed on one half the total eluate gave only slight color development, only a little more than is seen in the variation of one blank from another. Even so, this figure is taken as the maximum possible muramic acid in the L form. TABLE 3 compares the bacterium and its L form with regard to muramic acid content, as well as the other amino sugars found. It can be seen that the total amino sugar of

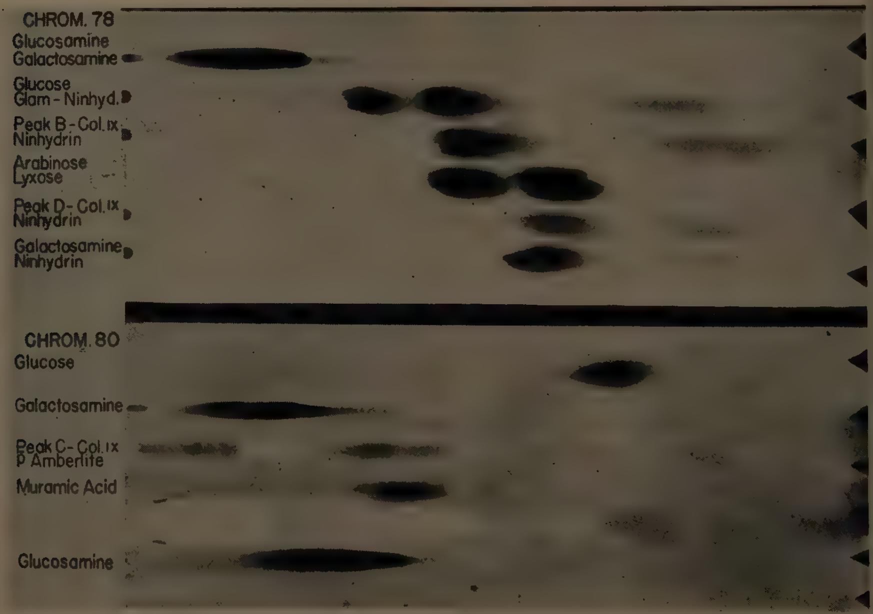

FIGURE 3. Paper chromatograms of amino sugars separated in column chromatogram IX. In paper chromatogram 78, where indicated, samples were degraded with Ninhydrin in capillary tubes prior to spotting.[29] Development was 1× descending for 40 hours in butanol:ethanol:water, 4:1:1. Paper chromatogram 80 was developed 2× descending, 24 hours each time, in butanol:pyridine:water, 5:3:2.

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* Authentic muramic acid was supplied by R. E. Strange and F. A. Dark.

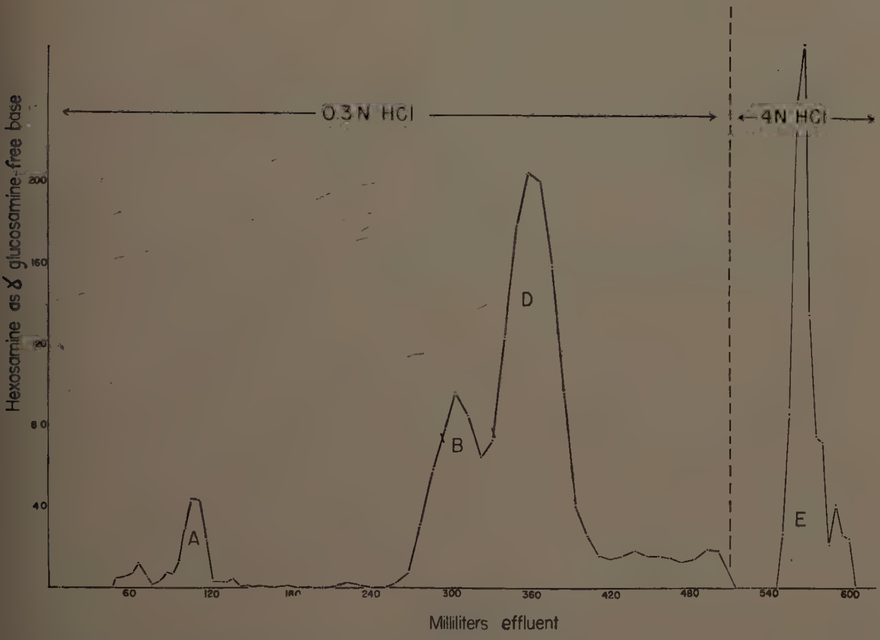

FIGURE 4. Hexosamine chromatogram XII (Dowex 50, 2 × 21.5 cm.). Hydrolyzate, acid-insoluble residue of *Proteus* 18 L, 892 mg.

TABLE 3

	Bacteria, 250 mg. Column IX	L Form, 1190 mg. of TCA-insoluble residue Columns XI and XII
Glucosamine	0.884 mg.* (0.353%)	1.472 mg.* (0.124%)
Galactosamine	2.702 mg. (1.08%)	4.328 mg. (0.364%)
Muramic acid	0.328 mg. (0.131%)	0.027 mg.† (0.00227%)
Peak A	0.218 mg. (0.087%)	0.247 mg. (0.021%)
Peak E	0.707 mg. (0.283%)	1.440 mg. (0.121%)
Totals	4.953 mg. (1.98%)	7.525 mg. (0.632%)
Muramic acid ÷ total hexosamine	0.067	0.0036

* Quantity of amino sugars obtained by summation of peaks in elution diagrams. Percentage of amino sugars related to dry weight of bacteria and dry weight of acid-insoluble residue of L form.

† Muramic acid not identified, but calculated as maximum possible muramic acid as determined by quantity of amino sugar not retained by Amberlite.

the L form is reduced to about one third of the value found for the bacterium. The various amino sugars are more or less proportionately reduced, except for muramic acid. When muramic acid is related to dry weight of organisms, it is reduced at least to one sixtieth of the amount found in the bacterium. If related to total hexosamine, muramic acid is reduced at least twentyfold.

These results are in agreement with Kandler's observation that a stable L form of *Proteus* did not contain an alkali-insoluble residue comparable to the bacterial basic cell wall component, the muramic acid-peptide compound. On the other hand, this study stands in contrast to the reports of Kandler's finding DAP and muramic acid, and Weibull's finding DAP in other strains of *Proteus* L forms. To what might these differences be due? The most obvious explanation is that these chemical differences reflect different types of L forms as a result of the kind or degree of chemical alteration. Such a conclusion would emphasize the differences that may occur between L lines, even of the same species, as already noted in morphologic studies.[15] However, this interpretation would exclude absence of the basic cell wall structure as a common denominator of the alteration occuring as a result of the transformation of a bacterium to an L form, and would require postulating either that there is no alteration common to all L forms or that the component altered is not muramic acid or DAP. Are there other possible explanations? Could the strains possessing DAP and muramic acid be penicillin-resistant morphologic variants similar to the strain studied by Ward? Of necessity, this remains a hypothetical question. It is posed here to emphasize the importance of further morphologic and biological study of L forms in conjunction with chemical studies on the cell wall components.

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CONTROVERSIAL ASPECTS OF THE MORPHOLOGY OF PPLO*

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Until the end of the 1930s the pleuropneumonia group (PPLO) appeared to be one of the best characterized groups of microorganisms. The strains were discovered by the association of two properties: filterability and ability to grow on artificial media. It turned out that all organisms discovered in this way have characteristic physical and morphologic properties in common. The sharp definition of this group disappeared in the early 1940s when it became apparent that under certain conditions bacteria grow without a rigid cell wall and become similar, in many respects, to PPLO. No single aspect of the bacterial forms usually called L forms indicates more strongly the similarity of these forms to PPLO than the length of time that was needed for the general acceptance of the fact that they derived from bacteria. For many years they were regarded as foreign PPLO mixed with the bacterial cultures.

The similarities and differences between PPLO and L forms are listed in TABLE 1. Before discussing these, it is necessary to indicate the information acquired about the nature of L forms and their derivation from bacteria. The evidence for the views presented is, to a large extent, morphologic and has been described in various publications.[1] Representative illustrations are shown in FIGURES 1 and 2.

Under the usual conditions of culture, bacteria reproduce, following moderate enlargement, by division into two. Certain conditions interfering with growth, such as the increase of acidity or the presence of small doses of penicillin, may induce the formation of long filaments. The growing units of bacteria multiply in the filaments and produce the usual cell wall, but do not separate from each other. The living reproductive units inside the cell wall can be characterized morphologically in some cases by staining the nucleotides, especially in filaments.[2] Under various conditions when normal reproduction is prevented, the bacteria grow into large round bodies, which in volume may be from a few times to several hundred times larger than the bacterium. These large bodies have aroused much speculation. Usually they are regarded as involution forms because they are produced under unfavorable conditions and usually do not reproduce when transferred. In favorable cases it is apparent that these large bodies are not produced by physical influences, such as imbibation of water, and that they are not produced by the gigantic growth of one living unit. These enlarged organisms are analogous to the filaments inasmuch as they consist of a few or a large number of living units of bacteria. They are different from the filaments in that the units are arranged tridimensionally and are not surrounded by the usual cell wall. The structure of the large bodies is apparent in appropriately stained preparations (FIGURE 1*f* and *g*) and is indicated by the

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ay in which bacteria are reproduced from the large bodies.[2] In some cases a ung large body, without changing its shape, divides by the development of ll membrane into a group of bacteria (FIGURE 1*b* and *c*). In other cases, cteria grow out of the large body in multiple directions (FIGURE 1*d*), or the ntent of the large body, without rupturing or deforming, is transformed into group of bacteria (FIGURE 1*h*).[3] While under certain conditions the large dy may produce the usual bacteria, under other conditions the living units of e large body may grow out in another form. This form can be kept in con- uous cultivation without producing a rigid cell wall. This is the L form of

TABLE 1

SIMILARITIES BETWEEN PPLO AND L FORMS OF BACTERIA

Both are simple organisms without apparent internal structure.

Physical properties such as softness, mechanical fragility, and instability in distilled water dicate lack of rigid cell wall.

Cultures of both contain organisms varying in size from small granules to large round dies.

Agar colonies of both have characteristic appearance and structure.

Response to antibiotics of both, especially resistance to penicillin, is similar.

Inhibition of growth by antibodies in the absence of complement occurs in both types of ganisms.

DISSIMILARITIES

L forms are not well adapted to grow on any of our present media and usually grow only der narrowly defined physical conditions. Few of the transferred organisms produce geny.

The colonies and the individual organisms in them are usually larger than those of PPLO.

L forms have been observed only in the laboratory, not as independent organisms in ture. PPLO are well established in nature.

Many strains of PPLO grow as well as bacteria on various media. The cultures are often pleomorphic than those of L forms. In the cultures of some strains, long branching filants develop.

bacterium. Transition from bacteria into the L form always occurs by wth of the bacterium into a large body, and the L forms always grow out m the large body. The large bodies may develop from bacteria and return bacteria in either solid or liquid media, but growth of L forms has been served only on agar medium, and the young L growth embeds itself into the dium. L forms grow in broth only occasionally, and growth occurs in large gregates: clumps or flocci. After they grow out from the large bodies, the ing units apparently need a support for growth.

These L forms are different in many respects from the usual bacteria. The wth is slow, although it can continue for a long time and eventually can duce large colonies. Both the initial growth from the large body and the tinued growth in media occur only under narrowly defined conditions in which the presence or absence of certain nutrients is not decisive. Some require very soft agar, some hard; some grow only within the agar in pour plates. Some need animal serum in the medium and some, high electrolyte or sucrose concentration. Usually no growth occurs in liquid medium, but the colonies on a piece of agar submerged in broth continue to grow. The organisms are soft and fragile, and are destroyed in the usual smear preparation. The slightest mechanical stress distorts them. They lack osmotic resistance and are destroyed in distilled water. The organisms in the L colonies are very pleomorphic, varying from small granules on the border line of visibility (FIGURE 1*i* to *o*) to large bodies similar to those from which they originated. They have a marked tendency to autolysis; the large bodies are transformed to empty blebs by gradual vacuolization, and the smaller forms lose their ability to reproduce and to be stained. The structure of the colonies on agar is characteristic. Even the smallest growth embeds itself into the medium, and growth continues mainly within the medium. After some development, the colony spills over the surface around the central zone and the characteristic "fried-egg" appearance is produced. On coagulated serum or egg, which is not penetrated, the organism develops only in a thin layer. The L forms resemble closely the parent bacterium in many properties, such as antigenic composition and metabolic activities. They behave similarly in the presence of antibiotics, with the exception of penicillin, to which they are entirely resistant. Whereas the basic morphology of all L cultures is similar, those obtained from different species differ from each other considerably in appearance, in growth requirements, and in their tendency to return to bacterial forms. Furthermore, a single bacterial strain can produce L cultures with markedly different properties. For example, *Proteus* and *Salmonella* produce two distinct types.[1] One of

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FIGURE 1. (*a* to *k*) The structure of the large bodies produced by bacteria, and their further development into bacteria and L forms. (*a* to *d*) The organisms on the agar surface a few hours after inoculation with a broth culture of *Bacteroides* strain 132. The bacteria were present in the broth cultures in the form of large bodies and short filaments with large central swelling. These forms are visible in *a*. In the upper right corner small granules adjoining the large body show the earliest stage in the development of the L forms. (*b* and *c*) The transformation of short filaments with central swellings into a number of bacteria. (*d*) The shape of one of the large bodies indicates growth into bacteria in multiple directions. A tiny colony of pleomorphic bacteria was produced from the other large body. (*e*) Branching filaments growing from a large body of a *Proteus* strain in the presence of penicillin. Photographs *a* to *e* from the surface of agar without staining; *a*, *d*, and *e* magnified about 1300 times; *b* and *c*, about 2000 times. (*f* and *g*) Chromatinic granules in the large bodies of *Bacteroides* strain 132. In *f* are large bodies that developed in broth. In *g* is a large body markedly increased in size following transfer to agar. Giemsa stain. ×3000. (*h*) Part of a tiny colony of *Escherichia coli* in which the bacilli either have autolyzed or have grown to swollen filaments and large bodies. These structures are densely filled with bacteria of regular shape. No bacilli can be seen outside of these structures on the agar. ×1000. (*i* and *j*) The earliest stage of the development of L forms in *Proteus* 4 hours after inoculation of a plate containing penicillin with the bacillus. Note the small size of L granules. ×3000. (*k*) Growth of a tiny L colony in one direction. ×2000. (*l*) L forms of *Staphylococcus* after 4 hours' incubation. Note the small size of the granules. The elongated shape is the result of stretching the soft inoculum. ×2000. (*m*) A tiny L colony of a *Flavobacterium* showing the small size of the granules and their growth in one direction. ×3000. (*n*) Young L-type growth of streptococci: small granules and a few short filaments. ×2000. (*o*) Broth culture of L forms of *Salmonella typhosa* immediately after transfer to agar. The culture consisted of small granules that developed inside of large bodies. ×2000. (*p*) Pleomorphic *H. influenzae* on the surface of agar. ×2000. Photographs *h* to *p* from stained agar preparations dried on the slide. FIGURE 1*a*, *d*, and *m* reproduced by permission from the *Journal of Bacteriology*.

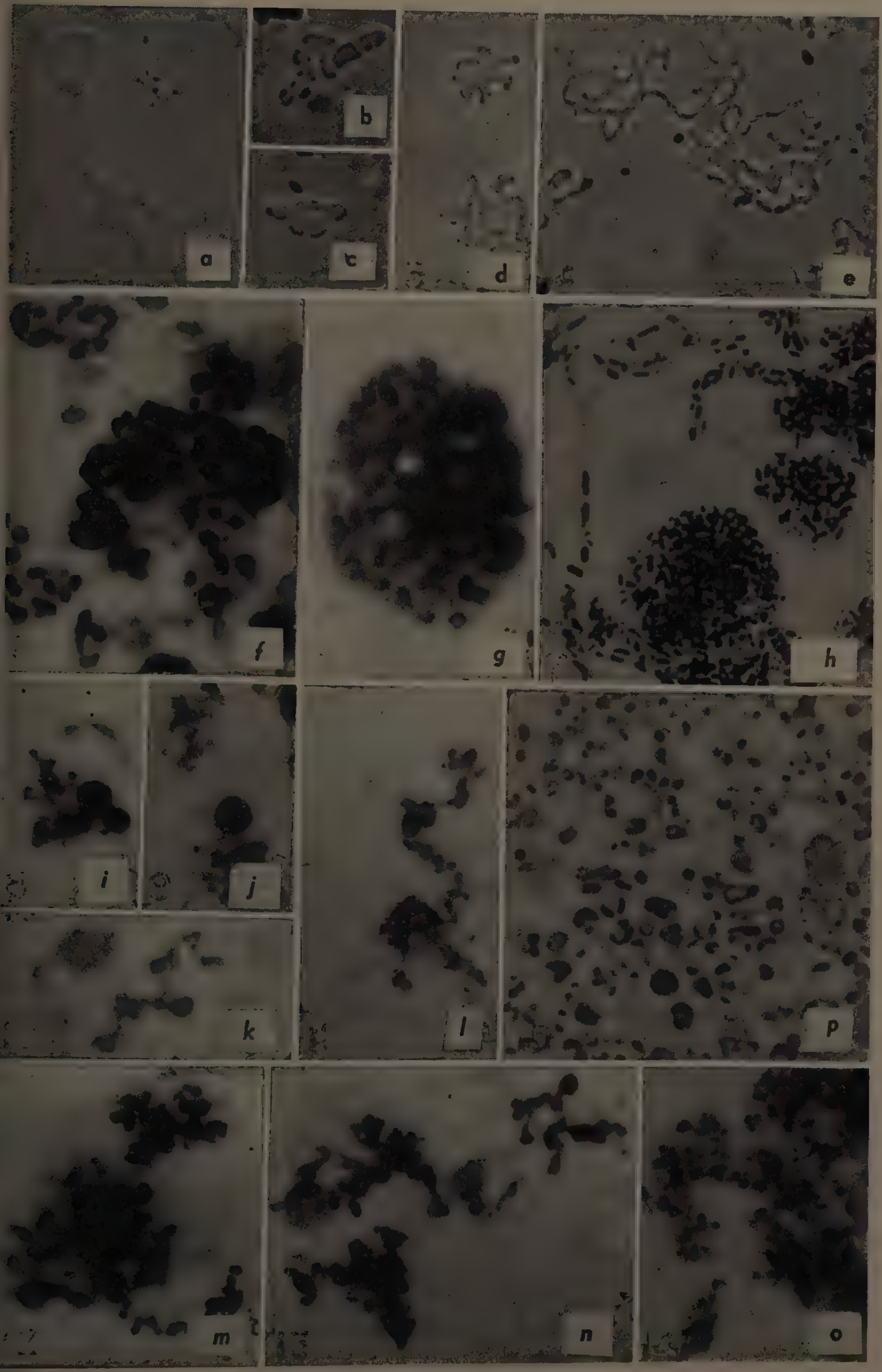

Figure 1.

these, designated as 3B, grows faster and produces larger colonies than the other type (3A), and the organisms are more deeply stained and less fragile. Strains of the 3B type grow well without animal serum in pour plates but do not grow at all, or only with difficulty, on the surface of the medium. If penicillin is eliminated, this type, without apparent growth in the L forms, reproduces the bacteria within a few hours. The 3A type grows more slowly; the colonies are smaller and require animal serum for growth. This type grows well on the surface of agar plates; it reverts to bacteria only after one or several days of growth, or it may lack entirely the ability to revert. In both types further variations were observed. For example, the 3B type, while retaining its morphologic properties and rapid growth, can lose completely the ability to return to bacterial form. There is a noticeable variation also among the L strains isolated from *Streptobacillus moniliformis*. Each strain has a distinctive appearance, with slightly different morphology and growth requirements. Observations of this sort indicate that bacteria can survive as L forms with more or less alteration of their structure and functions. The derivation and physical properties of L forms indicate that they represent the growth of the living units of bacteria without the usual cell membrane or with a greatly altered membrane. Studies with the electron microscope support this conclusion.[4] Recently the term protoplast has been proposed for the L forms.[5] The meaning of this term is too uncertain to be applied to the L forms.

It has been difficult to obtain information on the morphology of the small granules growing out of the large bodies other than that they are pleomorphic and of small size. They are arranged tridimensionally and embedded in the medium. When they are examined directly on the agar either stained or unstained, the growth of these granules presents a characteristic picture, but the shape of the individual organism is only rarely visible. The best pictures are obtained by a method similar to one used in the study of fungi and based on drying a stained agar preparation on a glass slide. The slightest manipulation of the cultures or contact with liquid distorts the organisms or completely destroys them. In successful preparations it is apparent that the size of the organisms growing out from the large bodies varies considerably (FIGURE 1*h* to *o*). They are sometimes as large as 1 to 2 μ, but in many cases they are at the border line of visibility (0.3 to 0.5 μ). That these small forms multiply is

FIGURE 2. (*a*) Electron micrograph of *Proteus* L forms growing as a film on the surface of broth. Unshadowed. ×6000. (*b*) Same as *a* but slightly shadowed. ×14,000. (*c*) Electron micrograph from a human PPLO strain. Unshadowed. ×6200. (*d* to *j*) PPLO strains of human origin. (*d*) Young broth culture. Giemsa staining. ×2500. (*e*) Earliest growth (8 hours) on the agar surface. Unstained. ×3000. (*f*) Two small PPLO colonies, 6-hour growth. Dried stained agar preparation. ×3000. (*g*) A young colony (8 hours) on agar. Agar fixation and Giemsa staining. Note the development of small elongated forms resembling bacilli. ×3000. (*h* and *i*) Preparations from 8-hour- and 24-hour-old agar cultures. Note the presence of tiny bipolar stained forms resembling bacilli. Agar fixation and Giemsa staining. ×3000. (*j*) A PPLO colony grown directly from the specimen. The surface is made up of large bodies, some of which are empty and some of which are filled with granules. On transfer from this culture, the large bodies developed into colonies. Stained agar preparation. ×900. (*k*) Young growth of the goat strain K5. Note the large size of the growing organisms. Dried stained agar. ×1350. (*l*) Same organisms as in *k* transferred from broth culture to agar. Note the pleomorphism and the presence of granules in the enlarged forms. Stained agar preparation. ×1350. FIGURE 2*c*, *g*, and *h* reproduced by permission from the *Journal of Bacteriology*.

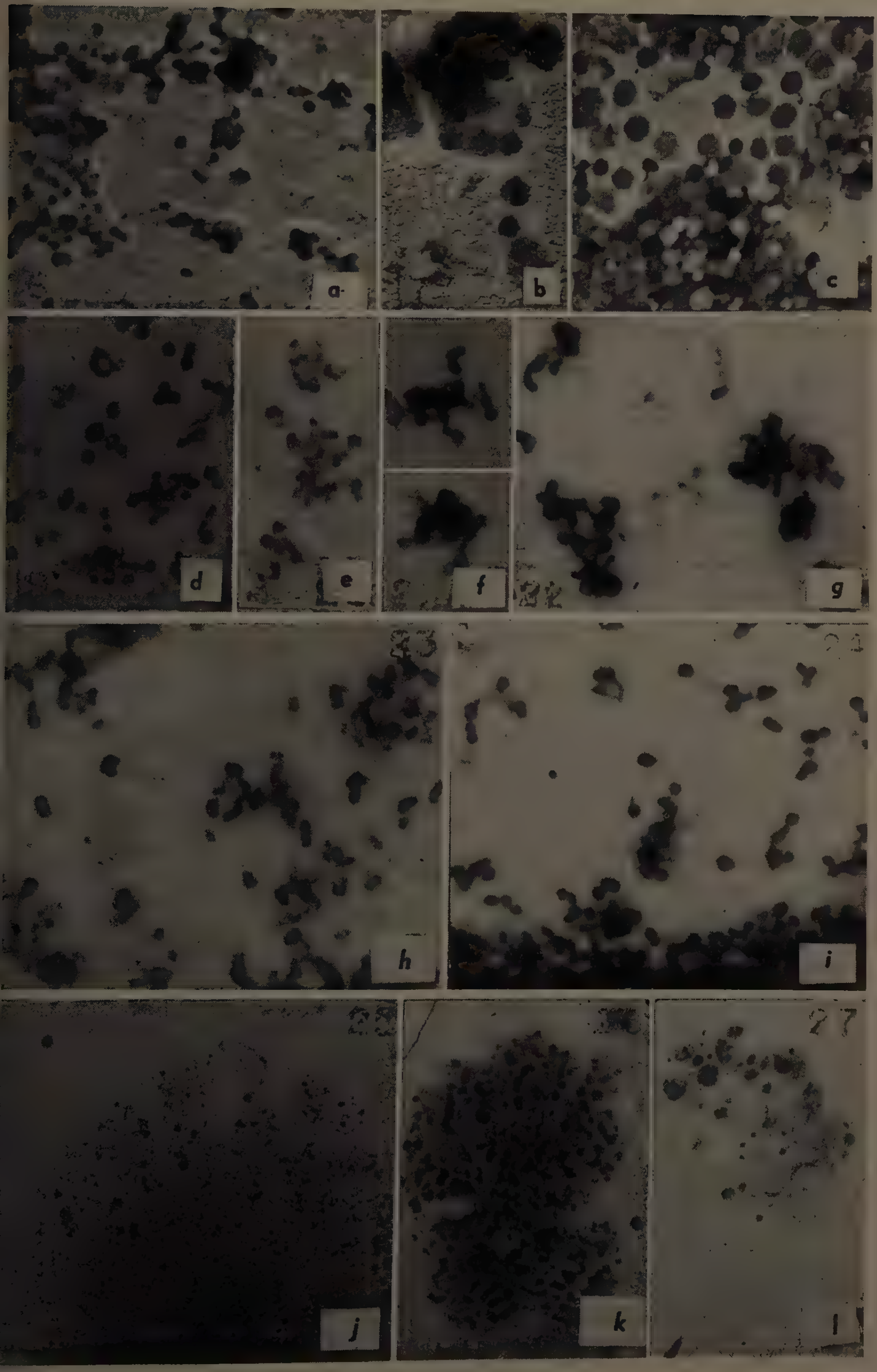

FIGURE 2.

indicated by the structure of their growth. The small forms penetrate into the medium in single rows or in branching strands. Sometimes the granules are deeply stained; at other times they stain only faintly. Two small granules are often seen close together; occasionally they are connected on both sides by a thin wall, presenting the appearance of a tiny bipolar stained bacillus. The length of such forms may be more than 1 μ and hence well within the resolution of the microscope. A few of these rodlike forms may be connected in a short straight filament. Occasionally, at the beginning of development these bipolar forms are seen in small groups apart, but later they rarely are seen among smaller and larger organisms. It is uncertain whether their similarity to bacterial forms indicates a true bacterial structure.

It is surprising that the soft fragile organisms of the L cultures are able to penetrate the agar. Lederberg[5] suggested that the soft mass of the growing organisms herniates into the meshes of the agar gel. The smoothness of the boundary of the large organisms and the structure of the small colonies are not in agreement with this supposition. Only active growth can produce the arrangement of the small organisms in rows or in branching strands. The suggestion that multiplication is by budding[6,7] cannot be applied to the smallest forms, whose arrangement indicates reproduction by binary fission.

The organisms in all L forms have the tendency to grow to large round bodies, and in many L cultures most of the organisms are in a transitory stage of this process. For a while the large bodies remain highly refractile and stain deeply. Later they are transformed by vacuolization into empty blebs. At this stage they show no sign of reproduction and are evidently dead. However, while they are full and can be stained, their reproduction can be observed. In transplants made from certain cultures, only the large bodies start to reproduce; in some cases, the small forms grow to large bodies before starting to reproduce. It is especially significant that in 3B L-type colonies the bacterial growth starts exclusively from the large bodies. In a recent publication of Thorsson and Weibull[4] electron micrographs from sections of the large bodies of *Proteus* L cultures show that some large bodies are filled with granules of about 0.2 μ similar to granules outside of them. Granules of similar size are present in sections of *Streptococcus* L forms.[8]

Early development of the organisms into large forms and autolysis are indications that the L forms are not well adapted to growth and survival in our media. This is especially apparent in the poor growth of transplants. Growth usually starts only from a mass transfer, and only a very few organisms, one in many thousands or millions, produce progeny. The need for support by agar gel or by high electrolyte or sucrose concentration points in a similar direction. The L forms of known bacteria, except for one case which will be mentioned later, have never been encountered in the natural habitat of the bacteria. Our information on the L forms is still meager; our impression will probably change, but it does not seem unlikely that the L cultures now propagated in the laboratory are artificial structures comparable to the tissue cultures of animals and plants. These structures are interesting in many respects, but there is no evidence to show that, in the form in which they are known, they play a role in the life of bacteria. On the other hand, it is unlikely that similar considerations apply to the large bodies produced from bacteria. These are encountered in the natural habitat of the bacteria, as in the mucous membranes and in the feces. When the large bodies develop, increase of the living mass continues for a long period under conditions in which individual bacteria cannot multiply. After a slight change of conditions large bodies formed in this way return to the bacterial forms. It is unlikely that such a process plays no role in the life of bacteria.

The distinction between bacterial and L-type growth is always clear-cut. Multiplication occurs either in bacterial form, which may be more or less pleomorphic, or in forms of soft fragile granules with a tendency to grow into the agar. However, the bacteria exposed to influences that induce transformation into L forms as, for example, sub-inhibitory doses of penicillin, show morphologic changes indicating weakening of the cell wall. They grow to filaments and swell to large bodies. Multiplication in such pleomorphic cultures occurs in the bacterial forms and not in the forms of soft granules characteristic of L forms. When fully developed, the colonies may consist exclusively of large round bodies, but these develop from bacteria and do not multiply in the original colony. These large bodies are soft and fragile, and they have weak cell envelopes. When they are transferred and return to bacteria, at first they often produce bizarre pleomorphic forms and, in some cases, long branching filaments (FIGURE 1*e*). Pleomorphism and branching in the filaments are consequences of the weakened cell wall that allows disorientation of the growing units. The tendency to grow with a weakened cell wall is apparent in some cases by the ease of the transformation into L forms and the return from these back to bacterial forms as a result of slight changes in environment. Some *S. moniliformis* strains grow in the bacillary form in broth. Transferred to agar after a few divisions, the bacilli swell to large bodies, and growth continues in the L form. These L forms, transferred to broth again, grow as bacilli. Strains of various bacteria isolated from pathological processes are inclined to pleomorphism and to spontaneous transformation into L forms, which indicates that bacteria in pathological processes may have a natural lability of cell wall formation. Despite this, during twenty years I have observed only one case in which it appeared probable that the bacteria were present in the cells of a pathological process in the L form.[2] The fact that L forms of *S. moniliformis* were cultivated directly from rats or humans does not necessarily indicate that they were present in the specimen in such form.

There is no reason to believe that transformation into L forms is a genetic change, even when the tendency to resume bacterial form is lost. Under appropriate conditions almost all bacteria show the first stages of L transformation, and the bacteria recovered from the L forms show no genetic change. The 3B-type L forms that Lederberg studied behave in conjugation like the bacteria from which they originated. There is no similar information on the 3A type, which do not return easily to bacterial form. In microorganisms, including the Hyphomycetes, changes in properties produced by outside influences may be transmitted indefinitely during asexual reproduction. Transformation of bacteria into L forms, according to the present information, belongs to this category.

In the majority of PPLO strains the important morphologic characteristics are similar to those of the L forms. They are, as in the L forms, the consequence of the absence of a rigid cell wall in an organism otherwise possessing potentialities for growth similar to those of the bacteria. PPLO present a structure as simple as that of bacteria. The larger forms in the cultures appear to be aggregates of small ones and show no signs of higher organization, such as are seen in fungi or protozoa. Their physical properties, their variation in size and the nature of their pleomorphism, are comparable to those seen in the L forms. The appearance and structure of agar colonies are similar. The properties that distinguish them from the L forms probably can be explained as the result of a higher degree of adaptation to life in this form. There are individual differences between different strains in both groups, and their properties are influenced to a large measure by the conditions of culture. Among the PPLO, the organisms in the avian and saprophytic strains are noticeably less fragile than the others and are usually of a larger size.[9] This variation in morphology is more marked in the L forms, and an experienced observer usually can differentiate them from PPLO. However, the differences between the two groups are quantitative, with gradual transition and overlapping of the properties.

One of the marked quantitative differences between PPLO and L forms is in filterability.[10] Filtrates of L forms through fritted glass filters retaining bacteria are usually sterile. In the few cases where their size could be determined by filtration, the smallest viable granules in L forms were found to be somewhat larger than those of PPLO and to make up a much smaller fraction of the viable organisms. However, in contrast to most viruses, the filterable fraction of the viable organisms is also small in PPLO. In addition, there is a great variation in filterability between cultures of the same PPLO tested on different occasions. Both with the light microscope and the electron microscope[6,9,11-13] it is apparent that the size of small organisms of various PPLO and L forms is often similar (FIGURE 2*a* to *f*). Electron micrographs from a *Proteus* L strain show masses of small granules comparable in size to the small organisms of PPLO (FIGURE 2*a* to *c*). In older colonies of L forms, corresponding to their greater tendency to pleomorphism and autolysis, the size of the organisms is usually much larger than in PPLO. The result of filtration is influenced in a large measure by the state of dispersion of the organisms and by the viability of single organisms. In both respects, the L forms are less appropriate for filtration. For this reason, microscopic observation gives a better impression of the distribution of different-sized organisms in the cultures. The difference in size between the two groups would have to be much greater to be of differential importance. It should be noted especially that there is no indication in the microscopic pictures that small forms are produced in different ways or have a different significance in the two groups.

Small rodlike bipolar stained forms such as those described in young colonies of L forms are also visible in PPLO;[14] they sometimes produce short straight filaments (FIGURE 2*g* to *i*). These elements in the young colonies come nearest in appearance to mycelial growth. They cannot be isolated by culture from the other elements and are connected with them by gradual transition of forms. It seems that the granules have a tendency to keep a bacillary form, which is counteracted by their small size and by the rounding up of the organisms. Preparations from pleomorphic *H. influenzae* strains (FIGURE 1*p*) occasionally give a similar impression; in addition to small biopolar stained bacilli, small and large round forms increasing to the size of large bodies can be seen in the preparations. All of these forms are fragile and easily distorted. The phenomenon mentioned earlier, in which certain bacteria, when transferred to agar, are transformed after a few divisions into L forms, also may be pertinent. In such cases it would be only one step further in the same direction for bacteria to assume the properties of PPLO. This behavior has been observed on several occasions, with small Gram-negative bacteria inhabiting the mucous membranes and isolated from pathological processes. This is the origin of the suggestion that PPLO may be related to such bacteria and may represent a further stage in adaptation to parasitism.

It is apparent that PPLO are better adapted to grow in culture than are the L forms. In contrast to L forms: if a fresh culture of PPLO is transferred to appropriate media and examined after 6 to 12 hours, almost every granule has begun to form a colony. In broth many PPLO strains produce a heavy growth of discrete organisms. The single organisms are as viable on various media as bacteria. There are, of course, exceptions. Some PPLO strains can be kept growing only with difficulty, while the old L1 strain of Klieneberger-Nobel,[10] which is also the most easily filterable L form, grows well on agar. Prolonged growth in uniform small granules and the development of pleomorphism only near the end of the growth period, as observed in PPLO strains, is not a characteristic suggesting essential differences; it merely indicates a better adaptation of PPLO to media.

Concerning the large bodies, the comments made in reference to L forms apply to PPLO also. In bovine pleuropneumonia strains, it has been described repeatedly that, after transfer into fresh broth, the granules increase in size and germinate in multiple directions forming the so-called asters.[15] It is apparent that the increase in size parallels the development of multiple centers of growth. Such a process cannot be regarded as degenerative. Development of granules inside the large bodies and the start of growth in transplant from the large bodies have been observed in many strains. The concept proposed by Klieneberger-Nobel is the most reasonable explanation for the development of the large forms. The elementary units remain together after division and form larger aggregates which, by change of conditions, may separate at any stage of development. These aggregates are produced under conditions unfavorable for growth, but multiplication continues in them and they survive longer than the individual units. The causes of inhibition of growth leading to increase in size and to the development of large bodies both in bacteria and PPLO are so variable that these processes cannot be the effect of a special type of injury. They must be the result of a specialized reaction of the organisms. When Freundt[15a] observed that the cultures of bovine pleuropneumonia are killed by acid produced from glucose, it should have been determined whether the small or large forms remained alive longer. The full significance of the large forms is not known, but the information that we possess is not sufficient to cause us to discard them as degenerative forms.

The development of branching filaments in some strains of PPLO and their absence in the L forms is thought by Ørskov and Freundt[16-18] to represent a fundamental difference between these two groups. They regard these filaments and the small granules to which they disintegrate as the only characteristic elements of the cultures of PPLO. They base the definition of the whole group on these elements. The morphology of freshly isolated strains of bovine pleuropneumonia is impressive. Ørskov studied it in 1927, when only the bovine and sheep strains were known and before the L forms were discovered. The concept that he formed is one-sided and leaves out of consideration characteristics that are at least as important as growth into filaments. One of the main objections to it is that in most strains of PPLO filamentous growth cannot be observed at all or is so inconspicuous that competent observers studying their morphology have not seen these forms. Through the course of years I have repeatedly examined PPLO strains from mice, rats, goats, birds, and humans, and also saprophytic strains with special attention to the development of filaments without seeing filamentous forms. These forms have not been seen by Sabin,[19] Morton *et al.*,[13] Liebermeister,[9] or Kandler and Kandler.[6] In the photographs published by Cuckow and Klieneberger-Nobel[12] they are present only in goat pleuropneumonia, which seems to be closely related to bovine pleuropneumonia. The characterization of a whole group of strains having many important properties in common cannot be based on a single property that has been seen by experienced observers in only a very few strains.

Some of the observations on which the views of Ørskov and Freundt are based also merit comment. I have had no success in observing the development of mycelial structures in PPLO strains with the methods used by them, either by direct microscopy of agar cultures or by observation of developing colonies in slide cultures following the recommendations of Ørskov.[16,17] The youngest growth is clearly visible in the dried stained agar preparations mentioned above. The only elements in them showing some resemblance to mycelia are occasional straight filaments composed of rodlike granules. Similar elements are also present in young L colonies. In slide cultures of both PPLO and L cultures I observed the appearance of floating filaments near the cotton fibers. These were definitely artifacts. My impression is that the results obtained with this method need careful scrutiny. To some extent this criticism applies also to pictures obtained by electron microscopy.[18] The pictures published of bovine organisms doubtless represent genuine structures and a morphology similar to that described by earlier authors.[15] Concerning the observations with human strains, it is very disturbing that Freundt found two strains of bovine pleuropneumonia among his human strains and that his evidence for the exclusion of mislabeling of the strains is not sufficiently convincing. The filamentous structures in bovine pleuropneumonia can be observed with the light microscope, the use of which gives less chance of producing artifacts and distorting the elements than do electron microscope techniques. Since filamentous structures have not been observed in most PPLO strains, it is unlikely that they are a basic feature in the development of strains other than bovine pleuropneumonia. It is even more significant that growth into filaments is not a basic structural property of microorganisms. Almost all bacteria, under certain conditions, grow into filaments. Weakening of the cell wall, as has been indicated, favors filamentous growth and makes possible branching of the filaments, the swelling to larger forms, and the germination of these in multiple directions. These are the most marked characteristics of the strains of bovine pleuropneumonia. Even if we should find that a rudimentary mycelial growth is more often present than appears today, this would not place bovine pleuropneumonia strains in a group apart from organisms such as the L forms with which they share other important structural properties. It should be noted also that no one has used the development of branching filaments in cultures to characterize PPLO strains. The tiny colonies on agar or the broth cultures cannot be identified by these structures. The criteria by which PPLO are identified follow closely the directions given by Sabin[19] with the modification that the size of the viable granules is determined by microscopic examination rather than by filtration. The physical make-up of the organisms, the absence of the rigid cell wall, pleomorphism, small size, and the characteristic structure of the colonies define an exceptionally well-delineated morphologic group. The basis of these morphologic properties is probably the absence of a rigid cell wall. The PPLO stand apart in this morphologic group as they probably have become adapted to life in this form over a long period of time and have their place in nature among other microorganisms. On the other hand, most of the L forms known at present are poorly adapted to live in this form, and our present cultures may represent artificial products of the laboratory.

It does not seem reasonable at present to make a more elaborate definition of the PPLO group or to try to determine more accurately its standing among other microorganisms, nor is there any evidence at present for or against the possibility that organisms of the PPLO group derive from bacteria. The observations that have been offered in support of this theory are not conclusive. In the last two decades much has been learned about the structure of bacteria, and we expect to learn considerably more in the near future. The chief functions of the tentative conclusions that can be offered are to suggest directions for future study. In this respect, consideration of various possibilities and the awareness of analogies and similarities between different microorganisms are most important, while attempts with our present knowledge to arrive at more definite conclusions may be detrimental.

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THE PREVALENCE OF PLEUROPNEUMONIALIKE ORGANISMS

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A voluminous literature on pleuropneumonialike organisms (PPLO) has accumulated since the first report on these organisms was made in the 1930s. The organisms have been found by workers in every continent on the globe. A knowledge of the prevalence of PPLO is essential when attempting to associate these organisms with certain disease processes, when doing pathogenicity studies, and when working with mammalian tissues or cells and with chick embryos.

Since Dienes and Edsall[1] reported the first isolation of PPLO from the genitourinary tract of man in 1937, numerous reports have appeared on the prevalence of PPLO in humans. PPLO are found frequently not only in the genitourinary tracts of man, but also in other regions of the human body. PPLO have been isolated from the oral region, the intestinal tract, the skin, pyogenic processes in various regions of the body, the blood stream during puerperal sepsis, the spinal fluid when there was involvement of the central nervous system, the fluid from arthritic joints, and from inflamed conjunctivae.[2] There is little doubt about the communicability of some strains of PPLO from man to man and about the ability of some strains to produce infections in man if the proper conditions prevail.

Following the extensive studies by Nelson in the early 1930s with the organisms, then designated as coccobacilliform bodies, isolated from chickens, the PPLO have come to assume a prominent place among the list of agents infecting chickens and turkeys. Pigeons, parakeets, and ducks are additional avian species from which PPLO have been isolated.[2] The organisms are passed through the egg, which affords a means of maintaining the infection among flocks; when infected chick embryos are inadvertently used in viral and rickettsial work, contamination of these infectious agents with PPLO may occur.

The many reports by Nelson have shown that PPLO are very prevalent among mice. The organisms are found in the conjunctivae, the respiratory tract, and the brain. Epidemics of infectious catarrh and conjunctivitis caused by these organisms among colonies of mice maintained for experimental work may have serious consequences.

Klieneberger's report in 1935[3] on the national occurrence of PPLO in rats is the start of the current interest in these organisms and is responsible for the use of the term "pleuropneumonialike organisms" and the letter L in reference to organisms that grow in a certain characteristic colony form. PPLO have been isolated from the oral cavities of healthy rats,[4] from the respiratory tract in cases of catarrh and bronchopneumonia, and from the joints, lymph nodes, heart's blood, spleens, and brains of rats exhibiting polyarthritis. Certain strains of PPLO are associated with pyogenic processes in the rat.[5]

Saprophytic and parasitic strains of PPLO have been isolated from the bovine genital tract by Edward, from milk by Alström, and from the lungs of calves with shipping fever.[2]

PPLO different from the causative agent of contagious agalactia have been isolated in recent years from the lungs of sheep in North America.[2] Little work appears to have been done on the presence of PPLO in normal goats, but apparently three diseases in goats may be caused by PPLO. These diseases are the contagious agalactia, a contagious pleuropneumonia, and the recently described highly fatal disease among dairy goats in North America that is characterized by septicemia and arthritis.

Dogs are another species of animal that frequently carry PPLO and, as in the case with many other species of animals, the organisms are apt to be more prevalent if some pathological condition is present. Until the past decade information about PPLO has been slow in appearing. For example, there was a period of seventeen years between the report by Shoetensack of the presence of PPLO in the respiratory tracts of dogs and the report by Edward and Fitzgerald of PPLO in the canine genital tract. There is evidence that the canine strains as well as the human strains of PPLO may be transmitted venereally.

There are unpublished data showing that cats may harbor PPLO. The organisms have been isolated from the conjunctivae of domestic cats exhibiting conjunctivitis.[2] There has been one report on the finding of PPLO in the genital tracts of horses. Within recent years swine have been found to harbor PPLO in their nasal cavities.

PPLO have been isolated from the nasopharynxes of normal guinea pigs and from abscesses in guinea pigs. Rabbits and hamsters appear to be the only common laboratory animals from which PPLO have not been isolated. Also, saprophytic strains of PPLO have been isolated from decomposing organic material, from sewage, and from cattle.

Since PPLO is so prevalent in man's environment, it is not surprising that man also harbors the organisms. It is evident that the presence of PPLO must be kept in mind when studying the etiology of disease when working with experimental animals, or when working with mammalian cells.

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THE PRODUCTION OF L FORMS OF ENTEROCOCCI

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In 1954 Sharp[1] introduced a high-salt medium that proved to be very successful for the production of L forms of group A streptococci. By means of a modification of this principle, whereby the chloride was replaced by phosphate, we were able to grow L forms from alpha-hemolytic streptococci in about one fourth of the number of strains examined.[2]

A chance observation led us to investigate more thoroughly the influence of this medium on enterococci. All the strains used in this study were made available by the Bacteriology Laboratory of the Department of Medicine of our hospital and, with one exception, were derived from urinary infections. As there is still no uniform agreement concerning the enterococci as a group, it seems desirable to state that the following diagnostic criteria were applied for screening: (1) Gram-positive cocci, (2) fermentation of esculin, and (3) heat resistance (60° C. for 30 min.).

A total of 29 strains was studied, all of which belonged to group D. In general, the technique used in this investigation was the same as that described earlier[2] in the study of L forms of alpha-hemolytic streptococci.

L-type growth was observed in 19 of the 29 strains in the first series, where media were used with 0.2 M phosphate and 16,000 U. of penicillin. This phenomenon appeared to be reproducible: 4 L-forming strains were tried 20 times, and in 16 plates L colonies were seen. Twenty-six attempts were made with 9 negative strains, and in none of these cultures was there any L-type growth.

The morphology of the L colonies of the enterococci did not seem to differ materially from that of the L colonies of other groups of streptococci described previously.[1,2] The macroscopic and microscopic appearance was characteristic, with growth into the agar and the typical pleomorphism. The average size of the individual colony was about 150 μ, ranging from about 100 to 250 μ. Occasionally however, much larger colonies were observed, especially on the first culture in L form, when penicillin was added to the medium in a central trough. Classic L colonies developed around this penicillin reservoir, whereas the large colonies showed a preference for the region situated peripherally around the L-growth zone. These colonies consisted of a core that was indistinguishable from an L colony, but having an outgrowth of large ringlike structures. These structures were sometimes larger than 20 μ in diameter, and they seemed to be growing on the surface of the medium. Sometimes they bridged the area between colonies forming a structure like an irregular cobweb. It seemed to be possible to use this material for subculture in L form or reconversion into the coccal form in penicillinfree media, although it is difficult to exclude contamination by classic L forms. It must still be decided whether these structures represent the "large bodies," as intermediates between the coccal and the L-type growth, or whether they should be regarded as separate phenomena. Two of our strains, which did not produce L forms under the standard conditions, showed these large rings, and once these forms were seen together with typical L colonies on the same plate.

As heat resistance (60° C. for 30 min) was one of the main criteria used to select strains for this study, this feature was analyzed in the coccal as well as in the L form of the same strain and also after reconversion into the coccal form. All the coccal forms were heat-tolerant, but the L colonies lost their viability when treated in a similar way. To minimize the number of variables in this test, blocks of the agar medium—each with several colonies—were put into tubes containing a salt solution comparable with the one used for the plate, and these tubes were heated in a water bath. All attempts to use these blocks for subcultures were negative whenever they contained L colonies. It is necessary to realize that these results do not prove with certainty that the L forms of this series are heat-labile, as under the conditions of our experiments the heat influence could involve indirect effects, for instance, the denaturation of serum components of the medium which are indispensable for the normal growth of L forms. As it has not been possible thus far to obtain L-type growth of enterococci in a liquid medium, a direct approach was not feasible.

The grouping of the strains was done by the Fuller method, and all belonged to group D. Eighteen were hemolytic, the others nonhemolytic, but this characteristic did not have any bearing on the ability to grow in L form under the conditions of the experiments. Fourteen of the 18 strains that were hemolytic could be grown in the L form, compared with 5 of the 11 nonhemolytic strains.

Reconversion from the L form into the conventional form occurred every time in all the strains that grew in L form whenever the penicillin was withdrawn. These reconverted cultures had the same characteristics as the original strains: they fermented esculin and were heat-stable and Gram-positive, whereas the L forms were Gram-negative. The grouping yielded the same results as they did prior to the L growth, and the penicillin sensitivity fell in the same range, which was between 2 and 4 U./ml. To avoid the possibility of contamination of the L colonies with persisting coccal forms, these determinations were made only after four or more subcultures in L form. Thus far no attempts have been made to obtain so-called fixed L forms, which could be maintained in the L form in spite of the absence of penicillin. Even after 12 subcultures in the L form, reversion into coccal form was seen on the next transplant without penicillin.

The phosphate concentration of the media was usually kept at 0.2 M, but subculture was also possible when the concentration was lowered to 0.1 M. Higher phosphate contents did not seem to be more favorable for the production of L forms but, according to Schönfeld (personal communication), other anions have been used with success. Schönfeld obtained L colonies from three strains of enterococci by applying Sharp's original medium with 3 per cent NaCl.

The penicillin content of the medium appeared to be a far more critical variable for the inducement of L growth of these microorganisms. One of the problems we are at present engaged in analyzing may be defined as follows: Is the ability to grow in L form a property of all strains within a given species or is it a characteristic of a certain group of strains within that species? If the latter were true, it might be worthwhile to investigate the difference between the potential L formers and the strains that cannot be induced to grow with L-type growth. Thus far, available data indicate that the experimental conditions are the limiting factor. With a view to the importance of the influence of penicillin, this variable is being studied in more detail at present. Preliminary results justify further testing along these lines. One strain classified as negative was plated in its coccal form on the usual medium but, instead of the routine amount of 16,000 U. of penicillin, the central trough was filled with 32,000 U. after incubation at 37° C. for 1½ hours. Growth in L form was seen within the normal observation period of 7 days. Besides being negative under standard conditions, this strain was also negative in spite of this large amount of penicillin, when the incubation period prior to the addition of penicillin was either one half or two hours.

This points to a possible importance of the time factor, and we wonder whether the primary action of penicillin must not take place at an as-yet-unspecified moment of the growth curve. Of the remaining 9 negative strains, 6 were tested with 50,000 U. of penicillin and, of this series, 3 showed L colonies, thus increasing the number of L-type-forming strains to 23 of 26.

In summary, it may be stated that a large majority of enterococci could be induced to grow in L form in a medium containing horse serum and extra phosphate in addition to penicillin. Reconversion into the coccal form occurred regularly, and no differences were observed between these recovered strains and the parent strains.

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THE L FORMS OF *PROTEUS MIRABILIS*[*]

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Recent investigations on L forms and PPLO (Kandler and Zehender, 1957; Lederberg and St. Clair, 1958) have provided data that suggest the hypothesis that these forms represent bacteria with defective cell walls. The wall defect may result from inhibition of synthesis of cell wall material either by a genetic block (stable L forms and PPLO) or by the presence of inhibitors such as penicillin.

The present paper reports data on penicillin-induced L forms and "protoplasts" or "large bodies" of *Proteus mirabilis* and attempts to evaluate these data in the light of the above hypothesis.

METHODS

The organism used is a laboratory stock culture of *P. mirabilis*. Manipulation, assay, and growth conditions of the L forms have been described elsewhere (Medill and O'Kane, 1954; Medill-Brown and Hutchinson, 1957).

Media. Brain heart infusion broth (Difco) and agar were used for assay of rods and initial growth of rods from stock culture.

All other cultures of rods, protoplasts, and L forms with and without added penicillin were grown in the following media:

	Final concentration per liter
K_2HPO_4	9.0 gm.
KH_2PO_4	1.0 gm.
Sodium lactate	20.0 gm.
KCl	22.5 gm.
Vitaminfree casamino acids (Difco)	10.0 gm.
Glucose	10.0 gm.
Nicotinamide	1.0 μg.

Glucose was autoclaved separately and added before use. Agar, when used, was sterilized separately and added to a final concentration of 1.1 per cent.

Dilutions of L forms and protoplasts were made in M/3 sucrose.

Inocula. Rod growth was obtained by inoculation of growth flasks containing 100 or 1000 ml. of media to give a 1:100 dilution of an 18-hour culture.

L-form growth in liquid media was obtained by transfer of penicillin agar blocks (1000 U./ml.) containing L-type colonies (48 hours old) into broth containing penicillin (400 U./ml.). After 48 hours a 1:100 dilution of this was made into new penicillin broth. The latter was used for analysis after incubation.

Protoplasts were obtained by adding penicillin (final concentration, 400 U./ml.) to 5- or 6-hour growth flasks of rods. Analyses of these were made after 1- and 2-hour incubations.

All flasks were incubated at 37° C. on a rotary shaker.

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Chemical Analysis

Cell nitrogen. The digestion procedure of Umbreit *et al.* (1945) and the Nesslerization process of Vanselow (1940) were used. Color intensity was measured with an Evelyn colorimeter at 420 mμ.

Nucleic acids. Cold trichloroacetic acid (TCA) to give a final concentration of 10 per cent was used for precipitation of the cells. Ribonucleic acid (RNA) was extracted with cold 10 per cent perchloric acid (PCA) by the method of Ogur-Rosen (1950).

RNA was estimated by ultraviolet light absorption at 260 mμ and by the orcinol reaction of Dische (Chargaff and Davidson, 1955).

Desoxyribonucleic acid was extracted with hot 5 per cent PCA and measured by the diphenylamine test (Chargaff and Davidson, 1955; Burton, 1956), and by ultraviolet light absorption at 260 mμ.

Details of these procedures and comparisons with other methods are being reported by one of us (Cocklin, 1959).

Reducing sugar was determined after hydrolysis with 1.3 N HCl (1 hour at 100° C.). The methods of Nelson (1944) and Somogyi (1945) were used.

Ether-soluble lipid was measured by the extraction of lyophilyzed cells with ether. The ether was evaporated to dryness, and the residue was weighed.

Phosphate was measured from TCA extracts and residue by the method of Fiske and SubbaRow (Hawk *et al.*, 1947).

Cell volume was measured by centrifugation of the culture in calibrated tubes.

RESULTS

General characteristics of protoplasts and L forms. The characteristics of protoplasts and L forms are listed in TABLE 1. The L forms are more resistant to osmotic shock than the protoplasts and more viable in liquid media containing penicillin.

Characteristics of the L forms of mutant strains of *P. mirabilis* (Medill, 1954) are contrasted with the rods in TABLE 2. The L form in each case exhibits the mutant character. The L forms are also similar to the rod in nutritional requirements and urease production. They differ in viability, osmotic fragility, and sensitivity to inhibitors found in natural media ingredients (Medill and O'Kane, 1954).

Numerous attempts were made to obtain biochemically deficient mutants of *P. mirabilis* in order to use ones pertaining to cell wall components for study of L forms. Following ultraviolet irradiation, the surviving cells were subjected to the standard isolation procedure for auxotrophs (Lederberg and Zinder, 1948; Davis, 1948) with negative results. The ingenious technique of Lederberg and St. Clair (1958), which has resulted in the isolation of mutants of *Escherichia coli* that have lost the ability to form diaminopimelic acid (DAP) and hence are stable L forms, was also employed with negative results. These experiments are being continued by Violet Breckbill of this laboratory.

Chemical studies of rods, protoplasts, and L forms. The production of nucleic acid and nitrogen during growth of *P. mirabilis* rods can be seen in the typical experiment presented in TABLE 3. Upon the addition of penicillin at 6 hours the nitrogen continues to increase, but at a slower rate than in the control. There is no reduction in the amount of RNA that has reached a peak at 6 hours in the control. DNA continues to increase at about the same rate as in the control. When penicillin is added at 5 hours, when the RNA is still increasing, the increase continues at the same rate as in the control.

TABLE 1
CHARACTERISTICS OF PROTOPLASTS AND L FORMS OF *P. MIRABILIS*

	Protoplasts	L forms
Morphology	Spherical	Spheres, irregular forms and granules. The elements tend to be more spherical in broth than on agar. About 0.2 to 7 μ in diameter.
Motility	Motile	Motile. Flagella may be seen by use of dark phase contrast.
Viability	Revert to rod form when removed from penicillin. Eighty per cent grow into L-type colonies if plated on penicillin agar. No further growth if transferred to penicillin broth.	Revert to rod form when removed from penicillin. Will produce L-type colonies if transferred onto penicillin agar. Will grow as L form if transferred to penicillin broth.
Osmotic fragility when suspended in distilled water	Survival: 0.05 per cent	Survival: 30 per cent

TABLE 2
COMPARISON OF THE RODS AND L FORMS OF MUTANT *P. MIRABILIS* STRAINS

Strain		Sucrose fermentation	Glucose fermentation	Growth on streptomycin 1000 μg./ml.	Growth on sodium azide M/500
K*	Rods	Slow†	Fast	0	0
	L forms	Slow	Fast	0	0
K/CV/az	Rods	Slow	Fast	0	+
	L forms	Slow	Fast	0	+
K/S	Rods	Fast‡	Fast	+	0
	L forms	Fast	Fast	+	0

* K is the wild-type strain.
† Three to 4 days before appearance of acid.
‡ Acid production within 18 hours.

The cell volume of protoplasts increased at a greater rate than in the control (TABLE 4). The larger cell volume of protoplasts was observed in many experiments. Dry weight measurements showed no difference between rod and protoplast.

Reducing sugar content of hydrolyzed cells, total phosphate, acid-soluble phosphate, and ether-soluble lipids showed no significant difference between rod and protoplast.

L forms grown in liquid media for 48 hours are low in nitrogen in comparison with rod cultures of comparable age. The RNA per N, DNA per N, and RNA per DNA ratios are within the range exhibited by rod cultures.

Development and reversion of L forms. The development of *P. mirabilis* L-type colonies from single rods (Valentin, 1957) is essentially similar to the same processes described by Freundt (1950), Höpken and Bartmann (1955), and Lederberg and St. Clair (1958). Rods develop irregular protuberances that penetrate the agar and multiply by "pinching off."

TABLE 3

COMPARISON OF NUCLEIC ACID PRODUCTION OF RODS, PROTOPLASTS, AND L FORMS OF *P. MIRABILIS*

Hour	μg. N/ml.	RNA/N	DNA/N	Viable count/ ml. × 10^8	RNA/DNA
Rods					
1	5.3	0.366	0.246	0.6	1.5
3	20.5	1.820	0.185	3.1	9.8
5	75.7	1.160	0.102	18.7	11.3
6	87.0	1.150	0.178	20.8	6.4
7	111.5	0.830	0.186	26.6	4.5
Protoplasts					
7 plus penicillin*	92.9	1.060	0.204	20.4	5.2
Rods					
12	156.1	0.750	0.230	58.3	3.3
18†	155.8	0.764	0.246	44.4	3.1
48	222.7	0.817	0.420	131.0	1.9
				Viable count/ ml. × 10^5	
L forms					
48 plus penicillin*	25.0	0.650	0.220	1.1	3.0

* Four hundred U./ml. added at the sixth hour.
† Inoculum.

TABLE 4

COMPARISON OF CELL VOLUME OF RODS AND PROTOPLASTS OF *P. MIRABILIS*

	Control rods		Protoplasts in penicillin
	6 hrs.	7 hrs.	7 hrs.
Ml. of volume/10 ml.	0.005	0.009	0.012

The reversion of the *P. mirabilis* L forms which have been removed from penicillin to rods has been described elsewhere (Medill and Hutchinson, 1954). Spherical forms elongate, branch, and eventually divide into rods.

P. mirabilis swarm cells never develop into L-type colonies, although they may produce large bodies (protoplasts) both with and without the presence of penicillin. The microscopic observations on this point were confirmed by making replicas on penicillin agar from velvet impressions of swarming colonies on agar without penicillin. This was done at intervals throughout the swarming cycle. In no case were L-type colonies formed on the replica plates from areas on the original plate showing a majority of swarm cells. The small colonial cells and the small rods resulting from the division of swarm cells, however, produce L-type colonies readily when impressed on penicillin agar.

DISCUSSION

The chemical data presented do not reveal a possible cell wall defect of *P. mirabilis* L forms and protoplasts. However, other workers (Kandler and Zehender, 1957; Weibull, 1958a; Lederberg and St. Clair, 1958) have shown that defective cell walls of stable L forms may have smaller amounts of hexoses, hexosamine, and diaminopimelic acid (DAP) than normal rods. *E. coli* mutants that fail to synthesize DAP grow as stable L forms unless DAP is present in the media. On the other hand, penicillin-induced L forms appear to have as large amounts of the above components as the rods.

The chemical data presented here do not show any effect of penicillin on nucleic acid production, although nitrogen production may be slightly inhibited. Other workers have reported some inhibition of RNA production by penicillin (Kandler and Zehender, 1956), and loss of RNA when protoplasts are obtained by lysozyme digestion (Fitz-James, 1958).

The morphologic observations on the development and reversion of *P. mirabilis* L forms can be interpreted as a loss and restoration of cell wall rigidity.

In light of the failure of swarm cells to develop into L-type colonies on penicillin agar, it is interesting to review former data on reversion of L forms (Medill and Hutchinson, 1954). At one stage of the reversion of L forms that have been removed from penicillin agar, almost 100 per cent of the reverted rods develop into cells that are morphologically identical to swarm cells. If such cells are placed on penicillin agar, they fail to develop into L-type colonies. If transferred to new media without penicillin, they divide into small rods that will produce L-type colonies when inoculated on penicillin agar. When penicillinase is added to L forms before reversion, they never go through the "swarm" cell stage, and the reverted rods will develop directly into L-type colonies if placed back on penicillin agar.

The protoplasts and L forms of *P. mirabilis* are motile. Other workers report lack of motility, although flagella may be present (Lederberg and St. Clair, 1958; Weibull, 1958*b*).

Some interesting comparisons can be made on the L forms of *P. mirabilis* and those of *E. coli* (Lederberg and St. Clair, 1958). The stimulating effect on L type colony production by *P. mirabilis* of increasing the penicillin concentration (Medill-Brown and Hutchinson, 1957) was also found to operate in the *Escherichia coli* system. *P. mirabilis* L forms grow readily on the surface or subsurface of agar media, while the *E. coli* L forms will only develop in pour plates. The *E. coli* L forms derived from streptomycin-resistant strains were sensitive to streptomycin. In contrast, the *P. mirabilis* L forms derived from resistant strains were fully resistant to the drug. Finally, *P. mirabilis* has thus far proved refractive to techniques for the isolation of auxotrophs that have been successful with *E. coli*. *P. mirabilis* protoplasts and L forms are motile, while those of *E. coli* are not.

The cell volume of protoplasts is always greater than the normal control, while the increase in protoplasmic constituents and dry weight is no greater than in the control. This might indicate an intake of water by the protoplasts.

Although similar in some respects, the protoplasts differ from L forms by a greater sensitivity to osmotic shock and by the absence of multiplication in liquid media.

SUMMARY

Chemical and cultural comparisons are made of rods, L forms, and protoplasts of *P. mirabilis*. The latter two forms were produced under the influence of penicillin.

The only definitive difference found among the three forms is in morphology, osmotic fragility, and capacity for reproduction.

L forms in liquid media are similar in some respects to protoplasts, but differ in their greater osmotic stability and their ability to reproduce in liquid media.

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DISCUSSION OF PART I

Edwin M. Lerner, II (*National Institutes of Health, Public Health Service, Bethesda, Md.*): It would appear necessary to accept some absolute classification, such as that proposed by Edward and Freundt, despite some of the objections raised by those who disagree with an arbitrary and possibly even artificial scheme. Most workers in this field, or indeed in any biological area, will agree that some sort of practical taxonomic scheme, incomplete as it might be and with all its inherent disadvantages, is preferable to no scheme at all. It is essential that we all know what we are discussing and writing about; without a generally accepted means of identifying these microorganisms, the confusion already existing in some quarters would be further confounded.

Certainly, a definite distinction between the L forms ("L-phase variants") of bacteria and the PPLO (Mycoplasmatales) should be made at the present time, especially in light of the several reports on "transformation (or reversion) of PPLO into bacteria" and "reversion of PPLO into L forms." Whether the above-mentioned phenomena are valid, a common language of description would aid immeasurably in dispelling some of the confusion engendered by such reports. Whether the PPLO constitute a biological group or order, distinct from the L forms of bacteria, an operative descriptive system will be of value in the resolution of such a question.

It is not essential that the taxonomic criteria, such as morphology, staining properties, growth requirements, fermentative abilities, immunologic properties, and chemical composition, withstand the test of time. These criteria and others have been universally applied to the taxonomic definition of well-known members of the Eubacteriales, and have been shown to break down, singly or severally, in many cases. However, the demonstrated fallibility of some of these criteria has not been considered grounds for discarding them. It is essential that all available criteria be employed to distinguish each PPLO, L-phase variant (stable or unstable), protoplast, and spheroplast, and to classify it, albeit in arbitrary fashion, so that those working with the same or different microorganisms may know for themselves, and make known to others, whether identity between cultures in different laboratories exists.

In this respect, the views of Klieneberger-Nobel that similar growth media, staining techniques, means of observation, and other such means be employed, at least *when describing one of these microorganisms taxonomically*, seem to be justified. Again, to draw an analogy from the bacteria, one certainly would employ the classically described media, staining methods, and other means for a particular member of the Eubacteriales when attempting to establish its taxonomic locus. Admittedly, the requirements of some species may be more exacting than those in common usage; however, a known member should be capable of demonstration by recognized and standard techniques.

In the course of reporting on, and hearing of, the many interesting and important individual features of this group of microorganisms, little has been said to emphasize the general biological interest and the significance implicit in them. This is perhaps due to an assumption that workers in other areas are generally aware of the unique properties shared by many members of this group.

We probably fail to realize that many biologists, and indeed some microbiologists, are not familiar with the fact that there exists a group of microorganisms that have not only such properties as extremely small size (smaller than the largest viruses and rickettsiae), filterability, and pathogenicity but, unlike the viruses and the rickettsiae, can be cultivated in a nonliving, cellfree environment. The ability of these minute, filterable units to reproduce themselves and produce whole colonies under such conditions should attract the interest of many more investigators and persuade them to explore the morphology, chemical composition, activities, pathogenicity, and pure biological significance of this borderline group of microorganisms that may conceivably bridge an evolutionary gap between the bacteria and the viruses.

Part II. Isolation and Propagation

INTRODUCTORY REMARKS

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I open this session with a brief historical note of local concern. Bovine pleuropneumonia, which is indirectly responsible for the present gathering, was supposedly first introduced into the United States in 1843 by a sick cow that was sold in Brooklyn, N.Y. This event was the beginning of an interesting era which ultimately led to one of the most massive attempts to eradicate an animal disease that was ever undertaken. The attempt was successful and, in 1892, a proclamation was issued by Jeremiah M. Rusk, then Secretary of Agriculture, declaring the country free from bovine pleuropneumonia.

ISOLATION AND IDENTIFICATION OF PPLO*

Sarabelle Madoff

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The first strains of the pleuropneumonia group of organisms were discovered in 1898 by the cultivation of filtered material from bovine pleuropneumonia. While sharing the property of filterability with the viruses, the ability of these organisms to grow on cell-free media did not prevent early workers from classifying them with viral agents. It was not until the late 1930s, after Klieneberger[1] had noted the association with *Streptobacillus moniliformis* of organisms resembling those of bovine pleuropneumonia, that interest in this unique group of organisms was stimulated. This L_1 form, as she designated it, was isolated and propagated on agar medium. Indeed, in the following years, both saprophytic and pathogenic strains resembling those of bovine pleuropneumonia were isolated on agar media from a variety of sources including man, animals, sewage, soil, and vegetable matter. It soon became apparent that all these strains, in addition to their small size, had other important properties in common. The organisms are very soft and friable, they are pleomorphic, and their size varies from the border line of resolution by light microscopy to several μ in diameter.

The conclusion that the L_1 of *S. moniliformis* was a variant of the bacillus was based on the demonstration of its reversion to the parent bacillus.[2] With the discovery that L forms could be isolated from many other bacteria, the distinction was made between those forms isolated from nature as pleuropneumonialike organisms (PPLO) and those derived from bacteria in the laboratory: the L forms of bacteria.[3]

More recently, increasing interest has attracted many new workers to the study of the pleuropneumonialike organisms. However, to the uninitiated observer, not only is the identification of PPLO difficult, but their distinction from L forms may be confusing. In our opinion, the difficulty that the newcomer may encounter in the isolation of these interesting forms lies, principally, in their initial recognition on agar media. It is for this reason that, in presenting the methods used in our laboratory for the study of PPLO, we emphasize their recognition and identification in agar cultures.

Cultivation

The primary prerequisite for the cultivation of PPLO is a good basal medium, to which must be added the essential growth factors. These essential growth factors are contained in fresh animal serum, both protein and lipid fractions being required.[4-7] These are best supplied by the addition to the medium of 10 per cent horse serum or 20 to 30 per cent ascitic fluid, the latter being the

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enrichment of choice in our laboratory. In most cases, primary culture on agar gives more reliable results and a greater percentage of positive cultures, as well.

Preference cannot be given to any particular basal medium, as every medium we have used has occasionally failed, for no apparent reason. Good results have been obtained with various beef heart infusion bases either freshly prepared or commercially manufactured. The most satisfactory medium in our experience has been that prepared in the laboratory from a tryptic digest concentrate of beef heart muscle.[8,9] The basal medium is adjusted to *p*H 7.8, and agar is added in a concentration of 1.5 per cent.

The final medium is prepared following the method of Klieneberger-Nobel[10] for "boiled blood" medium, with the difference that after precipitation with blood the medium is clarified by sedimentation. The effect of this procedure is to eliminate from the basal medium inhibitory substances rather than to add nutrient. It is our impression that a good basal medium is not improved further by precipitation with blood.

The procedure is as follows. To 100 cc. of sterile basal agar medium that has been melted and cooled to 50° C. is added 2 cc. of packed unwashed horse blood cells, and the mixture is brought slowly to a boil. The flask is placed in a water bath at 56° C. for approximately 30 min. until the precipitate settles; the clear supernatant is then decanted and ascitic fluid or horse serum added. The medium is poured into Petri dishes to a thickness of approximately 3 mm. Ascitic fluid should be adjusted to *p*H 7.4 with 3 per cent KH_2PO_4 immediately after collection, inactivated at 56° C. for 30 min., and checked for sterility before use. Each lot of ascitic fluid and horse serum should be tested for its ability to support growth of PPLO. Since the colonies of PPLO grow into the medium, the medium should be fairly soft with the final concentration of agar between 1.1 and 1.2 per cent. If horse serum is used, it is necessary to add an adequate amount of broth to obtain a sufficiently soft medium.

For primary isolation of PPLO from pathological material, the specimens are plated directly on agar medium by inoculating some portions of the plate heavily and others lightly. There appears to be no advantage to cultivation in liquid medium followed by transfer to agar. On the contrary, a disadvantage may be introduced by bacterial contamination of the broth. When heavy bacterial growth is anticipated from the specimen, penicillin is used to suppress such growth. Two methods are employed in our laboratory:

(1) A solution of penicillin is streaked with a glass rod over the surface of the medium to provide a few hundred units of penicillin per milliliter of the medium. By this means, growth of most bacteria is suppressed over the entire inoculated surface.

(2) The plates are first inoculated; then small troughs are cut out of the agar and a drop of penicillin containing 1000 U. is introduced into each trough. Bacterial growth is inhibited in a wide area around such troughs. Duplicate plates without antibiotic are always planted concomitantly. Both methods are simple, but when penicillin is used care must be taken to differentiate L colonies produced by bacteria from colonies of PPLO. This distinction will be discussed later.

Although pleuropneumonialike organisms are aerobic, on primary isolation growth is frequently obtained only under anaerobic conditions. We use Fortner's[11] method, which provides anaerobiasis as well as a moist atmosphere with CO_2 and has the advantage that each plate can be examined separately. It consists of sealing in with the culture a rapidly growing organism that uses up the oxygen. The specimen is inoculated as described. In the cover of the inoculated plate is placed a slab of agar from a blood agar plate (approximately one third of the plate). The slab of agar is then streaked heavily with *Serratia marcescens*. The Petri dish is then inverted into the cover, and the plate is sealed by filling the space between dish and cover with melted paraffin containing 10 per cent vaseline. To avoid overgrowth due to condensed moisture, all agar media should be dried in the incubator for a few minutes before inoculation. Best results with this method are obtained in a water-jacketed incubator where minimum variation in temperature prevents the formation of water of condensation. Plates are examined after 48, 72, and 96 hours' incubation at 35 to 37° C. Most strains of PPLO grow out in 2 to 4 days, but occasionally a strain will appear only after several additional days of incubation.

The typical large colonies of PPLO appear on the agar as minute discrete colonies with an opaque center and a translucent periphery. Tiny colonies cannot be seen with the unaided eye or with the hand lens. Transfer is made after microscopic identification. This is accomplished by cutting out a block of agar bearing colonies and streaking it over the surface of fresh agar medium. The agar block is left on the medium, as very often growth in transplant occurs only under the agar block. When only tiny colonies are found in the original culture, they may be very difficult to keep in subculture.

Microscopic Identification of PPLO

Definitive recognition of PPLO depends on the microscopic examination of the agar cultures. Several methods are in use, some providing only presumptive recognition of PPLO, others permitting final identification. Among the former are:

(1) Examination of the plates with the low power of the microscope (×100) and transmitted light. This method is reliable only if the PPLO colonies develop to a fairly large size and are well isolated from bacterial colonies. Tiny colonies growing out of human pathological material cannot be recognized in this manner.

(2) Edward[5] recommends the use of the dissecting microscope (magnification ×10) and slightly oblique transmitted light. This also requires considerable experience in order to distinguish PPLO colonies from other organisms.

(3) Examination of the cultures with phase contrast. This method requires a thin transparent medium that creates technical difficulties; even so, identification of small colonies is uncertain. It must be emphasized, however, that these methods are useful in examining large areas of plates and in the presumptive recognition of PPLO colonies.

Definite identification can be made only upon examination under oil immersion of suitably stained colonies. This may be accomplished by one of the following methods:

(1) The agar fixation method recommended by Klieneberger[12] and Salaman.[13] This method gives an excellent impression of the surface structures of agar

cultures and is the best technique for the study of the morphology of the individual organisms. However, its value for the primary recognition of PPLO is restricted by failure to demonstrate the structures growing into the medium; also, small colonies may not be recognized in such preparations. Furthermore, pleomorphic bacterial structures including large bodies and L-type growth may be indistinguishable from mature PPLO.

(2) The stained agar technique of Dienes[2] that is used in our laboratory. It is our impression that this is the simplest and most efficient method for the recognition and identification of pleuropneumonialike organisms. Colonies are stained *in situ* on the agar on which they were grown, and can be examined with the oil immersion lens. Even the tiniest PPLO colonies can be recognized in these preparations by their characteristic morphology and staining. Rare PPLO colonies can be found among bacterial colonies, and L-type growth arising from bacteria can be distinguished from them. Since we have used the stained agar technique almost exclusively for the identification of PPLO, the method will be presented in detail, and the appearance of the cultures stained by this method will be described.

Identification of PPLO by the Stained Agar Technique

The staining solution is made by dissolving 2.5 gm. methylene blue, 1.25 gm. azur II, 10 gm. maltose, 0.25 gm. Na_2CO_3, and 0.2 gm. benzoic acid in 100 ml. distilled water. By means of a cotton applicator, a thin film of staining solution is applied to coverslips (24 × 24 mm., No. 1) and allowed to dry. The coverslips are then cut into 4 squares with a diamond point pencil. Blocks of agar about 6 to 8 mm. are cut out of the culture and placed right side up on a glass slide. The dye-coated coverslip fragments are then placed stain side down directly on the agar blocks so that a projecting rim of coverslip extends on all sides of the agar. The space between the coverslip and the slide is then filled with melted paraffin containing 10 per cent Vaseline which, upon hardening, seals the preparation and permits examination under oil immersion without disturbing the coverslip. Staining is complete within a few minutes.

In such preparations, colonies of PPLO stain bright blue as do young viable bacterial colonies. Autolyzed bacteria, cellular material, and debris take on a pink hue. The agar stains faintly blue or violet. If the agar stains too deeply, it may be necessary to dilute the stain somewhat. If the agar is thicker than 2 to 3 mm. and not transparent, it may be necessary to examine a thin layer sliced from the top of the block by means of a razor blade. Even blood or chocolate agar treated in this way may give a satisfactory preparation. In selecting the block of agar to be examined, large bacterial colonies should be

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FIGURE 1. Photographs *a* to *e* were made from one area of a plate inoculated from the human urethra. (*a*) A streak of pus with very numerous tiny colonies of PPLO. In the less heavily inoculated area a small streptococcus is seen. Above the streak are visible a few pus cells and a moderate number of tiny PPLO colonies. There is no growth on the area below the streak. ×100. (*b*) Part of the streptococcus colony and the area to the left of it showing tiny PPLO colonies. ×900. (*c*) The surface of a heavily inoculated portion of the plate showing pus cells and dark areas produced by PPLO colonies below the agar surface. ×900. (*d*) The same preparation as in *c* in lower focus, showing the small PPLO colonies below the surface. ×900. (*e*) Same area as *c* and *d* with higher magnification. The structure of the colonies is apparent, and the individual organisms are visible. ×2000.

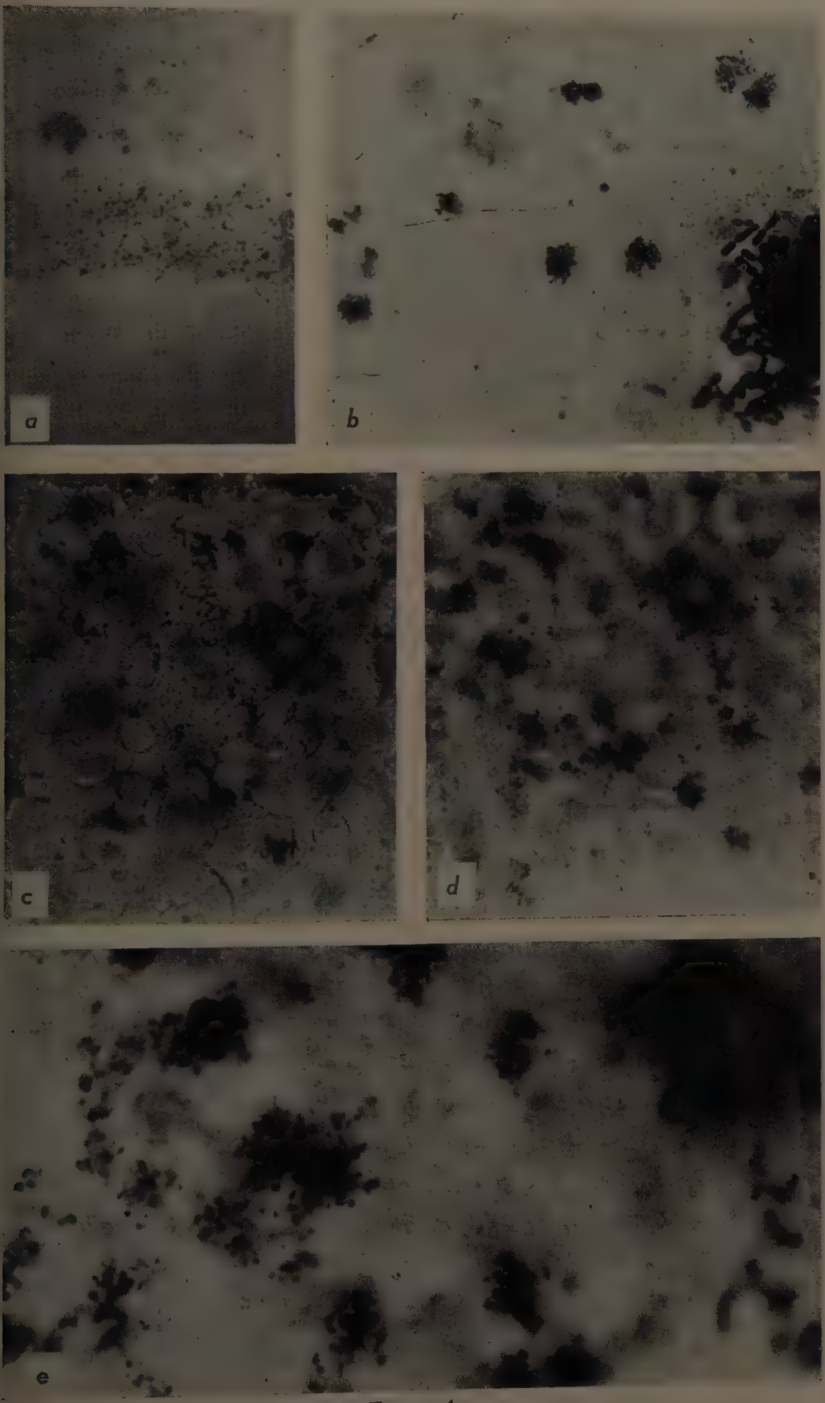

FIGURE 1.

avoided whenever possible, as they tend to decolorize the stain locally. This difficulty usually can be avoided by a brief exposure (approximately 20 sec.) of the block to the fumes of a loopful of concentrated HCl in a small Petri dish before staining. It is important to examine several preparations from a single culture from both heavily and lightly inoculated areas, as well as from areas adjacent to bacterial colonies.

It is hardly necessary to recommend that the detection of PPLO requires the use of a good microscope with a bright light diffused through ground or frosted glass. A low-power apochromatic objective (×10) with high numerical aperture is recommended for scanning, and ×15 oculars should be used for both low-power and oil immersion.

Under low power, most bacterial colonies have a smooth regular outline, and they stain evenly throughout. Large PPLO colonies, on the other hand, exhibit the characteristic PPLO structure with the center of the colony staining much more deeply than the periphery (FIGURE 2*a* and *b*).* Very young colonies of PPLO also take up the blue stain, and their outline is irregular (FIGURE 1*a*), but their final identification can be made only with oil immersion. This is especially true of very tiny PPLO colonies such as the T-form colonies described by Shepard.[9] It is a good rule to examine every tiny blue-staining colony that is not immediately recognized as bacterial with the oil immersion lens.

Although colonies of PPLO are small (from a few μ to 1 mm. in diameter) compared to normal bacterial colonies, the criteria by which they are differentiated from bacteria are independent of the size of the colonies.[14-16] These are: (1) Colonies of PPLO grow not only on the surface of the agar, but by penetration into the agar. The dense center of the fully developed colony is embedded in the agar (FIGURE 2*d* and *e*) and is surrounded by a thin peripheral zone on the surface, giving the colony the characteristic "fried-egg" appearance (FIGURE 2*a* and *b*). The center of the colony penetrating the agar stains much more deeply than the surface growth. Young colonies do not exhibit this appearance, but are distinguished by their irregular outline and by the presence of small pleomorphic granules whose shape is not clearly visible and

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* All photographs illustrating this article were selected from the collection of Louis Dienes. They were chosen with a view to illustrate the characteristic appearance of colonies of pleuropneumonialike organisms in cultures and to aid in their differentiation from bacterial colonies. All were made from stained agar preparations as described in the text.

FIGURE 2. (*a*) Well-developed PPLO colonies of a culture obtained from the human mouth. The colonies have a smooth appearance and consist of small organisms. Note that the center of the colony is deeply embedded in the agar. ×100. (*b*) Two PPLO colonies from a culture from the human urethra. One has no periphery. The other has a wide periphery consisting of large bodies, many of which are transformed to empty blebs. ×1200. (*c*) A small PPLO colony as seen with high magnification and focused slightly below the surface. Two tiny diphtheroid colonies consisting of a very few bacteria are visible on the surface of the medium. ×2000. (*d*) An area of a plate inoculated from the human genital tract. At the top of the photograph is seen the periphery of a staphylococcus colony adjacent to two small PPLO colonies. The lower part of the photograph is occupied by the periphery of a large PPLO colony. The surface of the PPLO colonies consists of large bodies, some of which are vacuolized. ×900. (*e*) Same as *d* with lower focus, showing the center of the colonies embedded in the agar. The irregular structure of the colonies and the small granules in them are characteristic. ×900.

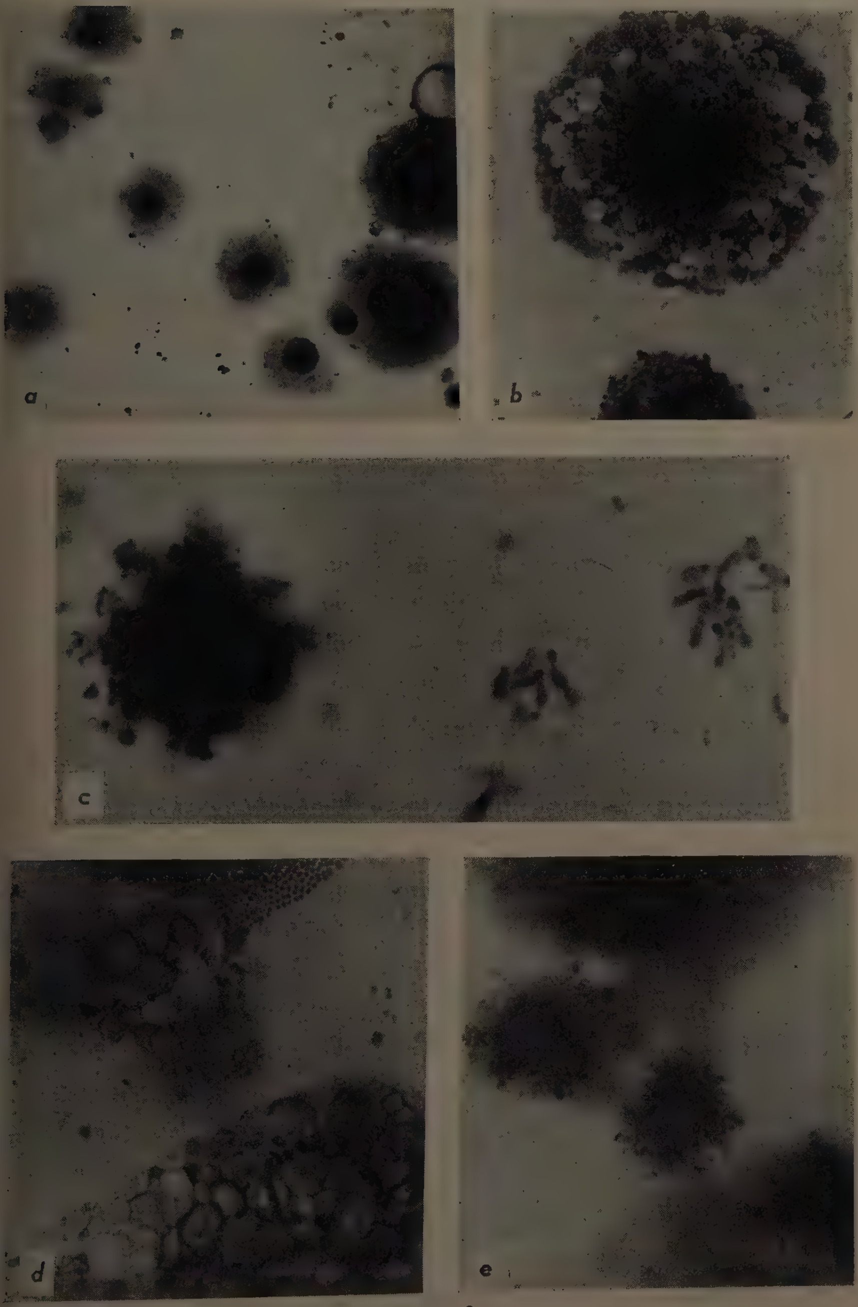

FIGURE 2.

which extend into the agar (FIGURE 1*b*, *d*, and *e*). Tiny colonies from pathological specimens are often located adjacent to or under cellular material, but proper focusing with oil immersion reveals their characteristic PPLO morphology (FIGURE 1*a* to *e*). On the other hand, tiny bacterial colonies grow on the surface of the agar, and their shape is often round and usually regular. On examination with oil immersion, the morphology of the individual organisms is clearly visible (FIGURE 3*c* and *d*). After two or three days' incubation, certain bacteria such as streptococci and some Gram-negative organisms may produce tiny secondary colonies below the surface of the parent colony. The arrangement of these colonies and the fact that the individual bacteria are distinctly visible in them clearly differentiate them from PPLO. Actinomyces colonies are characterized by penetration into the agar of single branching filaments of bacterial appearance.

(2) The individual organisms in young PPLO colonies appear as tiny granules of indistinct morphology. Their size may be very small, from the border line of resolution by oil immersion up to several μ. They are not so sharply outlined as bacterial forms (FIGURE 1*e*).

(3) On the surface of mature PPLO colonies, the organisms swell up to large bodies of 5, 10, or even 20 μ in diameter (FIGURE 3*a* and *b*). The large bodies stain well at first; later they appear as empty blebs.

(4) Colonies of PPLO are extremely soft and fragile. Even in the most carefully made preparations, the colonies may become distorted and appear as a soft mass of pleomorphic elements. The organisms do not separate, but their shape may be deformed and drawn out into filaments. Individual organisms are then unrecognizable.

In summary, these are the characteristic properties which allow the identification of PPLO and their differentiation from bacteria: (1) growth of the colonies into the medium; (2) the presence of small pleomorphic organisms; (3) the formation of large bodies or varying size; and (4) the softness of the colonies that may result in distortion of the elements composing them.

If penicillin is used to suppress bacterial growth, care must be taken to distinguish PPLO from pleomorphic bacterial growth and L colonies.[3,17] In the presence of penicillin, many bacterial colonies such as those of *Hemophilus influenzae* and *parainfluenzae*, *Neisseria gonorrheae* (FIGURE 3*d* and *e*), *Escherichia coli*, and *Proteus* may remain very small, while their organisms become very pleomorphic and develop into large bodies.[17] This is a surface phenomenon, and it may readily be seen in the stained agar preparations that there is no growth of the colonies into the medium. In impression preparations, however, such structures may be very misleading, since surface structures remaining in the stained impression may appear entirely similar to PPLO. Furthermore, penicillin is effective in transforming many bacteria into L forms. For example, *S. moniliformis* planted on media containing penicillin gives a pure growth of L_1. Occasionally, on direct culture of human pathological material, L forms have been observed arising from streptococci, *Bacteroides*, or various Gram-negative bacteria. These L colonies (FIGURE 3*f*) can usually be distinguished from PPLO under oil immersion. All L colonies, even very small ones, stain much more deeply than colonies of PPLO. Fully developed L colonies grow

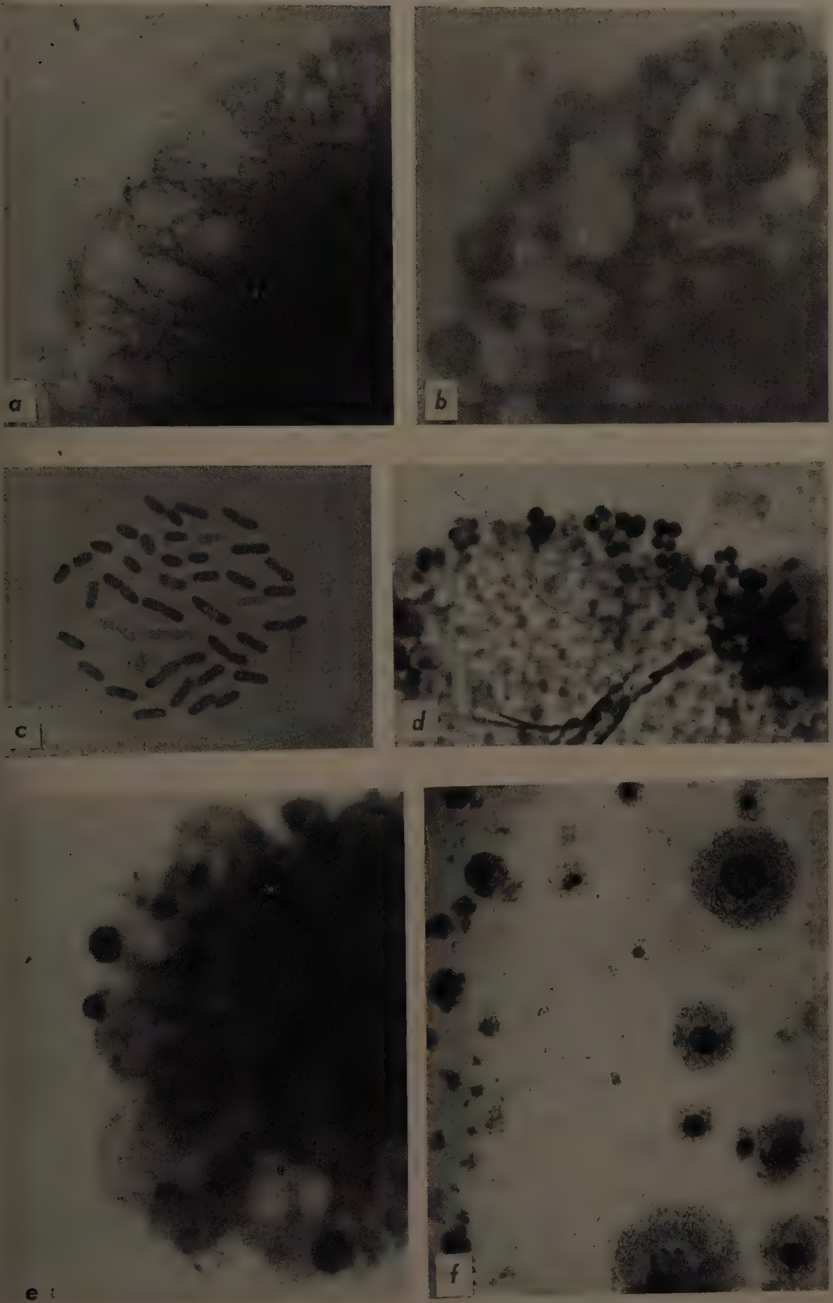

FIGURE 3. (*a* and *b*) Well-developed PPLO colonies from the human genital tract. Both vere photographed at the periphery of the colony at the surface of the medium. Note the lifference in the appearance of the large bodies in the two colonies. (*a*) ×2000. (*b*) ×1300. *c*) A young colony of *E. coli* photographed on the surface of the agar. ×2000. (*d*) The dge of a gonococcus colony photographed on the surface of the agar. ×2000. (*e*) A small onococcus colony in the presence of penicillin. The cocci are pleomorphic and in various tages of swelling up into large bodies. ×2000. (*f*) L colonies arising from an unidentified Gram-negative bacillus on a plate containing penicillin inoculated from human sputum. ×100.

to much larger size than those of PPLO, their structure is much coarser, and the organisms comprising them are of larger size. In the rare instance where an L form was indistinguishable from a PPLO on the original culture, its character became evident on transplant and subsequent study. We believe that we have never been misled in the identification of PPLO by their appearance on agar cultures stained *in situ* by this method.

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PROBLEMS IN THE ISOLATION AND IDENTIFICATION OF AVIAN PPLO*

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The diagnosis and control of chronic respiratory disease (CRD) in chickens nd turkeys are based upon the demonstration of the causative agents, pleuroneumonialike organisms (PPLO), or their antibodies in the tissues of affected irds. This paper is devoted primarily to the problems involved in the isolaion and identification of avian PPLO.

The etiological agents of CRD, which had been described previously as coccoacilliform organisms[1] or as viruses[2,3] were identified in 1952 by Markham and Vong[4] as PPLO. This finding soon was confirmed by many workers and led o a widespread interest in this group of organisms. However, several puzzling roblems soon emerged from the studies of the relationship of PPLO to CRD. ometimes it was found that PPLO isolated from cases of CRD failed to proluce symptoms or lesions of CRD, even when inoculated into the respiratory ract of susceptible turkeys. In other cases it was found that the disease could e transmitted with fresh exudates containing PPLO, egg-propagated strains f PPLO, or even PPLO propagated on culture media for a few passages. When ome of these organisms were carried for several passages on culture media, here was a rapid loss in pathogenicity of the PPLO. On the other hand, some PLO cultures retained a high degree of pathogenicity even after many pasages in culture media.

Early studies by Gianforte *et al.*[5] seemed to indicate that the PPLO isolated rom the avian respiratory tract fell into a single group on the basis of serologic nd biochemical characteristics. However, later studies[6,7,8] indicated that here were other and different PPLO strains in birds.

In 1957 Adler and Yamamoto[6] isolated two different strains of PPLO from he sinus of a turkey with infectious sinusitis. One of these strains grew readily n culture media and was not pathogenic for chickens and turkeys. The other lid not colonize readily on serum-enriched agar plates and was capable of prolucing disease. Additional studies by Adler *et al.*[7] demonstrated the existence f three serologically different strains of PPLO. These strains also differed in heir metabolic characteristics and their ability to produce lesions in turkeys.

The results of an extensive investigation of the occurrence, characteristics, nd significance of avian PPLO types were presented by Adler *et al.*[8] and by amamoto and Adler.[9,10] These studies indicated the presence of at least ive distinct serologic types that also differed in their physiological and pathoenic characteristics. Two of these types were apparently common and widely listributed. The first type, designated S_6, is pathogenic, highly antigenic, nd relatively slow-growing on culture media. This strain is the standard erologic type from which PPLO diagnostic antigens have been made. The

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second type, designated C, is nonpathogenic or weakly pathogenic, a poor antigen, and grows rapidly on culture media. Many other characteristics of these and the other serologic PPLO types were reported in this series of papers.

In an attempt[8] to evaluate the significance of these types, several series of PPLO isolations were made from birds with varied histories. In the first series, no PPLO were isolated from 50 chickens that were serologically negative to the standard antigenic strain (S_6) and free of clinical signs of disease. In a second series, PPLO were isolated consistently from 61 birds that were serologically positive to the standard antigenic strain but free of clinical signs of disease. However, all of these PPLO were found to be C-type organisms. In a third series of PPLO isolations made from 84 birds with signs of CRD, the organisms were found to be predominantly C-type PPLO; only five of these isolations were S_6-type organisms. These findings could be explained by the following observations:

(1) Mixtures of more than one type of PPLO are often found in cases of CRD. This was found to be true by workers at the University of California, Davis, Calif.,[6] and at Cornell University, Ithaca, N.Y.[11]

(2) Nonpathogenic or C-type PPLO tend to persist longer in the respiratory tract of birds than do S_6 types. This was noted in work at Cornell University (unpublished data).

(3) The present cultural techniques for the isolation of avian PPLO favor the growth of the C type of organism. The S_6 type of PPLO will usually grow on culture media, but not with the same rapidity and vigor as the C type. Adler and Berg (personal communication) found that, if mixtures of S_6- and C-type PPLO are inoculated and transferred in culture media, the C-type organisms will outgrow the S_6 type and become the predominant form in the culture.

(4) Some S_6-type PPLO strains are very difficult to adapt to culture media.[11,12]

As a result of these studies,[8-10] it became apparent that all of the previously described media isolation techniques[12-16] were of limited value. The interpretation of a PPLO isolation required study of the characteristics of the type of PPLO isolated. The effectiveness of media isolation procedures was complicated further by the difficulty in getting some strains of PPLO to produce colonies on agar plates[8] and by the variation in carbohydrate-fermentation patterns observed[8] in other strains.

Another problem was indicated by a series of experiments designed to test the pathogenicity of C- and S_6-type PPLO cultures in turkey poults. In these studies[8] it was found that the S_6-type cultures were uniformly capable of producing lesions. On the other hand, most of the C-type cultures did not produce lesions. Where lesions were found after inoculation with C-type cultures, the sera of the birds with lesions also contained S_6 agglutinins. This suggested that some S_6-type PPLO may have been present in some of the C cultures. When attempts were made to produce pure C-type cultures by serial single-colony picks and transfers, it was found that it was almost impossible to separate the two types by this method. A careful examination of the literature suggests that much of the previous PPLO work could have been done with mixed cultures, and that no reliable techniques exist for the production of pure

PLO cultures or for ascertaining their purity. These fundamental weaknesses tand in the way of present and future PPLO research. It is futile to discuss he interrelationships of the various PPLO types until techniques are devised or producing and identifying pure cultures of the organisms. It is equally itile to argue about the relationship of PPLO and L forms or to talk about he reversion of PPLO or L forms to bacteria for the same reason.

A further serious problem in avian PPLO studies is associated with the ina-ility of the present culture media to support adequately the growth of many athogenic strains of PPLO. In a series of comparative reisolation attempts vith PPLO cultures, Fabricant[11] has shown that the use of media or combina-ions of media did not give as good results as did yolk sac inoculation of chick mbryos in the reactivation of S_6 PPLO strains. On the other hand, no such ifference was noted in the reisolation of C-type strains. Further experiments n which reisolations were attempted from serial decimal dilutions of S_6-type ultures revealed that chick embryos were almost uniformly superior to media n the detection of this PPLO type. In some cases the use of chick embryos letected PPLO in dilutions two to three logs greater than the best of the media echniques.

Another source of confusion in the field of avian PPLO and, as I see it, in he whole PPLO situation, is the possible relationship of L forms of bacteria to hese organisms. Kelton and Gentry[17] have suggested that many so-called PPLO cultures consist of L forms of bacteria. This might indicate that the lifferentiation of L forms as well as of various PPLO types offers a problem n the isolation and identification of organisms from the avian respiratory tract. The reversion to bacterial forms of avian PPLO cultures has several possible xplanations: (1) bacteria are present in the original culture; (2) bacteria arise rom contamination during the reversion procedure; (3) the so-called PPLO ultures are made up of a mixture of PPLO and L forms; (4) some of the or-anisms termed avian PPLO are true PPLO and some are L forms; (5) all so-alled avian PPLO are L forms; or (6) several of the previous possibilities are rue.

The validity of any of these preceding hypotheses depends on an accurate nowledge of what is contained in the starting culture and on the definitions of PPLO and L forms. The solution to both of these problems depends, as I ave previously stated, on the ability to produce and identify pure cultures of he organisms we are studying.

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RECOVERY, PROPAGATION, AND CHARACTERISTICS OF T-STRAIN PPLO ISOLATED FROM HUMAN CASES OF NONGONOCOCCAL URETHRITIS

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Studies on the etiology of nongonococcal urethritis (NGU) in my laboratory have shown that a particular morphologic type of pleuropneumonialike organism is associated with the disease in approximately 70 per cent of over 500 cases of primary and recurrent NGU studied. Pleuropneumonialike organisms (PPLO) belonging to this particular morphologic group were designated T strains.

The procedure for isolation of T-strain PPLO from NGU patients has been described previously[1,2] and, in general, it has consisted of the following steps. Urethral exudate for primary culture was obtained by deep intraurethral scrap-ng of the urethral ceiling, employing a stiff, 22-gauge platinum inoculating oop, with the terminal loop bent at about a 10° angle. This technique for the collection of clinical material was employed both for obtaining material for culture and for the preparation of smears for study of epithelial cells. Culture plates were inoculated immediately, at the side of the patient, and were brought to the laboratory for planting of urine sediment and for preparation for incubation. All culture plates were incubated routinely at 35° to 36° C., employing the technique of Fortner[3] for the establishment of a microaerophilic environment. All primary culture inoculations were made directly to a solid, tryptic-heart-digest-ascitic agar medium, which has been described previously.[2] The highly characteristic colonies of T-strain PPLO generally reach full development by 48 to 72 hours. At this time they may be detected under the ow power of the microscope at 100 diameters by inspection of the inoculated area as visualized through the bottom of the culture plate. Confirmation of the low-power findings is accomplished routinely by examining wet-stained agar-block preparations under the oil-immersion objective, as recommended by Dienes *et al.*[4,5] It should be emphasized strongly at this point that proper employment of the latter procedure is, in my opinion, the only reliable method for the recognition and study of T-strain PPLO in primary cultures.

The successful propagation of freshly isolated T strains beyond primary culture has been and remains a difficult and challenging problem. My first attempts at propagation employed the same medium as used for primary isolation. Agar blocks were aseptically cut from 48-hour primary cultures bearing arge numbers of T-strain PPLO colonies and relatively free of bacterial colonies. Such agar blocks, bearing only T colonies, were inverted, transferred to fresh medium, and pushed across the surface of the medium to effect inoculation. These agar subcultures were prepared and incubated in the same manner as were primary cultures. The usual experience was to find all subcultures without evidence of multiplication, even after prolonged incubation. A very limited number of T strains, however, did show some inclination to grow in subculture on the primary isolation medium. Two T strains remained viable through 2 serial subcultures; 1 strain through 4 subcultures; and 2 strains were successfully subcultured through the fifth passage (14 days). All such T-strains were lost to further subculture. Furthermore, it was noted that the T strain colonies progressively diminished in size with each subculture. At the time of the last viable subculture, colonies were recognizable only with great difficulty, and were obviously moribund. Utilization of the primary culture medium for propagation purposes was abandoned.

I next turned my attention to the chick embryo in an effort to resolve the propagation stalemate. Eight T strains in primary culture from different NGU patients were subjected to propagation attempts in chick embryos, employing embryos of different ages and various routes of inoculation. Six of the 8 T strains failed to show evidence of proliferation after a minimum of 5 blind serial passages at 4-, 7-, and 10-day intervals. One T strain, however, showed evidence of limited multiplication in the fourth and fifth serial passages by the yolk-sac route, but failed to be detected in subsequent egg passages. Attempts to propagate freshly isolated T strains in embryonated eggs killed by chilling were without success.

The first successful propagation in serial passage of a T-strain PPLO occurred with the remaining T strain, No. 211B2. This T strain was recovered in March 1956 from an NGU patient with a history of intercurrent urethral discharge of 8 years' duration. Propagation was achieved in 7-day chick embryos inoculated by the yolk-sac route, and in 10-day embryos inoculated by the allantoic route. By the fourth serial passage, at 4- to 7-day passage intervals, T strain organisms were detected in the allantoic fluid and in yolk-sac fluid, and developed characteristic colonies upon subculture to solid medium. However, at this time continued subculture in artificial medium could not be maintained. Subsequent passages of this T strain were made by the allantoic route. Embryo death did not occur, nor was there apparent evidence of lesions or damage to the membranes. Allantoic fluid from the fifteenth passage was used to inoculate the minced embryo medium of Nelson.[6,7] Rapid multiplication of the organism occurred in the latter medium. Later, this T strain was adapted to cultivation in an artificial fluid medium devoid of cellular elements. Abundant growth occurred in fluid medium, although no detectable turbidity was observed until some time after the sixtieth serial subculture at 2- to 3-day intervals. To date, this T-strain PPLO has been carried through more than 373 serial subcultures in a tryptic-heart-digest-ascitic fluid medium. However, promising as this singular success proved to be, subsequent propagation attempts of additional freshly isolated T strains in chick embryos by the same technique yielded irregular, unpredictable, or negative results. In addition, all attempts at initial propagation of freshly isolated T strains in the minced embryo medium of Nelson,[6,7] or in other modifications of the Maitland-type[8] medium, also yielded negative results.

The above difficulties, compounded with the problems encountered in detection of early T-strain multiplication in embryonated eggs, prompted a search for a more satisfactory and reliable method.

Promising success in initial propagation recently has been obtained in monolayer cultures of explanted chick embryonic membranes. Three separate, eshly isolated T strains from different NGU patients have been propagated iccessfully in monolayer cultures of chick entodermal cells, after the technique escribed by Weiss and Huang[9] for the study of feline pneumonitis virus. Two f the 3 T strains propagated in chick entodermal cells have been successfully dapted to cultivation in artificial medium, both fluid and solid, and currently re being maintained in fluid medium. Both strains have been carried through 27 consecutive subcultures in artificial fluid medium, and continue to develop ighly characteristic T colonies when subcultured to solid medium. During iitial propagation in chick entodermal cells a dark phase was observed of from 4 to 28 days, representing 2 to 4 blind serial passages at 7-day intervals. Dur- ıg this phase no organisms were detectable by examinations of stained prepa- ations under the light microscope. A total of 9 to 12 serial passages was re- uired in chick entodermal cells before sufficient adaptation had occurred to ermit active growth when subcultured to artificial medium.

Initial propagation of freshly isolated T strains likewise has been attempted ı established cell lines of human epithelial-like cells, including a human skin pithelial cell,[10] a human synovial cell, strain McCoy,[11] and a human amnion ell, strain FL.[12] To date, all such attempts have met with failure.

The characteristics of T strain PPLO in primary culture are confined prin- ipally to colonial morphology on solid medium, and form the basis for recogni- ion and identification of the colonies. The most pronounced feature of T- train colonies is their extremely minute size at maturity, which generally is eached by 48 to 72 hours. Mature colonies range in size from 6 to 15 μ in iameter, rarely exceeding 20 μ. Due to their minute size, their recognition n primary culture plates may prove difficult when they are examined under ›w power (×100), as shown in FIGURE 1*a* to *c*. In wet-stained agar-block reparations examined under the oil-immersion objective, 48-hour T-strain olonies characteristically stain a light, bright blue with Dienes' stain.[4,5] Older olonies often stain poorly or remain unstained. The colonies are typically regular, roughly spherical, and grow downward into the agar medium without vidence of surface growth zones (FIGURE 1*d*). In structure, T-strain colonies re usually finely granular and consist of minute particles of uniform size, with- ut evidence of large-body formation. A less easily recognized colony form, hown in FIGURE 1*e* and *f* and designated the TR-type colony, consists of an regular mass of ramifying, fingerlike outgrowth of more loosely bound par- icles developing downward into the agar medium. The development of T- train colonies in primary culture is frequently closely associated with the resence of deposited epithelial cells (FIGURE 1*d*). This is especially true of 'R-type colonies, which have been observed developing only beneath deposited pithelial cells in primary cultures.

The characteristics of T-strain PPLO in propagation can be described best nder two separate headings: (1) initial propagation in an environment of ving cells, and (2) propagation of adapted strains. In the case of initial ropagation of T strains, we are dealing with the organism in tissue phase, in system of living cells. We are attempting to provide an environment some- 'hat akin to that found in the state of natural, clinical infection in the male uman host. In my experience, as pointed out, only a comparatively rare

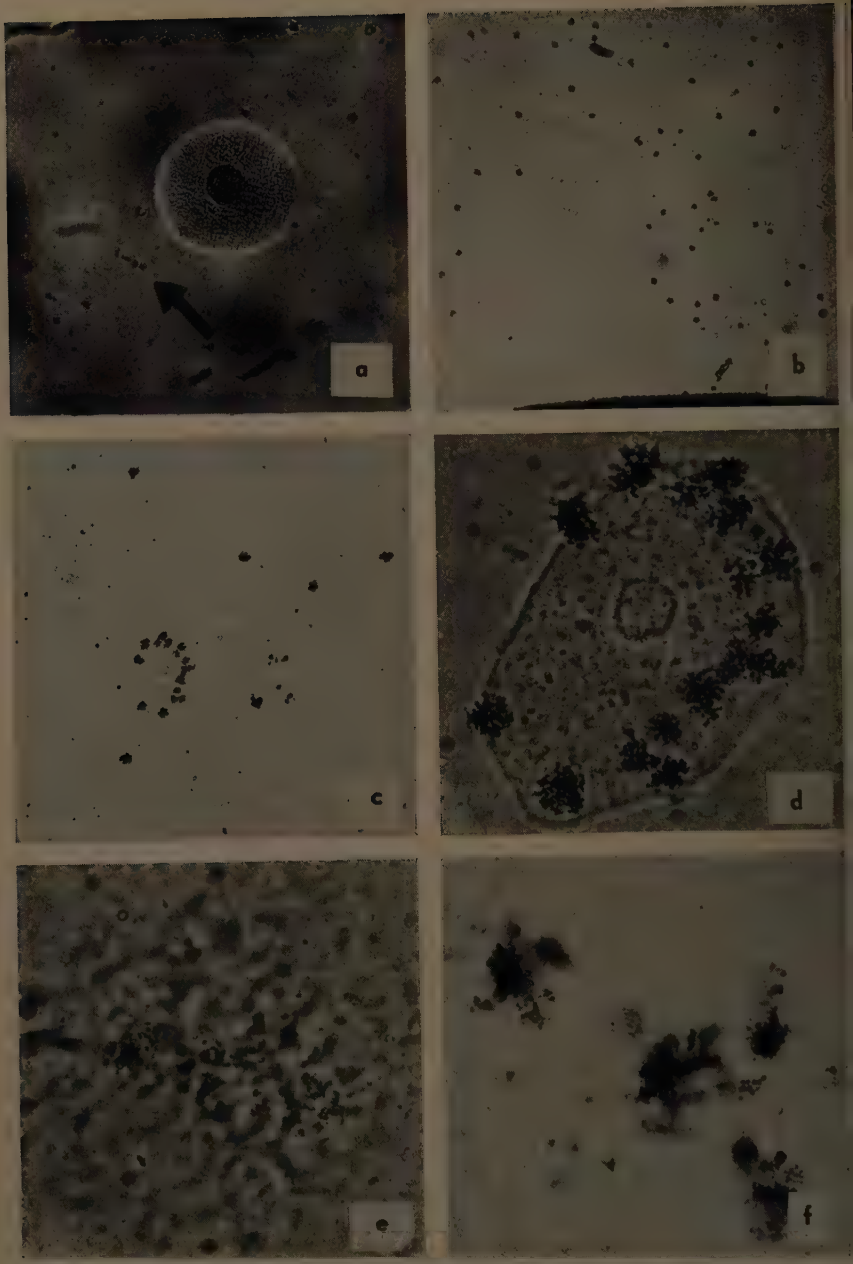

FIGURE 1. (*a*) T-strain colonies of human PPLO from nongonococcal urethritis patien Minute T colonies (one of which is indicated by arrow) are about 18 μ in diameter. Singl large, classic-type PPLO colony, included for size comparison, is approximately 325 μ in ameter. Primary culture, unstained, after 48 hours. Patient 162. ×85. (*b*) Small T col nies (10 to 12 μ) in large numbers in primary culture. Edge of a bacterial colony, shown the lower border, is included for size comparison. Wet-stained agar-block preparation. P tient 167. ×85. (*c*) T-type human PPLO colonies developing from a deposited epitheli cell. Six other T colonies are developing in the open area away from the cell. Prima culture. Wet-stained agar-block preparation. Patient 211B. ×180. (*d*) Same deposit epithelial cell as shown in *c*, but photographed at higher magnification to illustrate the natu and manner of development of the T colonies into the agar medium beneath the cell. P mary culture. Wet-stained agar-block preparation. Patient 211B. ×850. (*e*) and (TR-type, deep subcellular colonies. These are interpreted as variants of the T-type PPI colony. Primary culture, after 48 hours. Wet-stained agar-block preparations. Patien 106 and 232. ×850.

umber of T strains find the environment provided by embryonated eggs ap-ropriate. The environment provided by monolayer cultures of chick ento-lermal cells appears to provide more appropriate conditions for stimulating nitial multiplication of freshly isolated T strains. In addition, evidence of arly multiplication can be visualized easily in Giemsa-stained flying coverslip reparations of such cell cultures.

T strains in tissue phase in initial propagation in monolayer cell cultures resent a simple, direct picture. In my experience, the organisms have al-vays occurred in small to large aggregates of elementary bodylike, basophilic, ninute particles of uniform size and shape, as illustrated in FIGURE 2*a*. Their norphology is indistinguishable from that exhibited by the coccoid intracyto-lasmic inclusions seen in stained urethral epithelial scrapings from patients vith NGU (FIGURE 2*b*). During the dark phase, and the subsequent 29 to 62 lays in the adaptation phase, subcultures to artificial mediums were universally iegative. Commencing with about the ninth passage, active growth was ob-ained first in subcultures into artificial fluid medium and, later, after 2 to 4 dditional passages in cells, onto solid medium. In fluid medium, early cul-ures of adapted T strains produced no detectable turbidity, but consisted of arge numbers of single particles and small to medium-sized flocs of particles, eemingly embedded within a faintly staining matrix. Their staining reaction o Giemsa stain was slightly acidophilic. Subcultures of adapted tissue-phase rganisms to solid medium provided the first indication that the monomorphic coccoid) structures seen in infected cells in culture were, indeed, T-strain PPLO. Highly characteristic, minute (8- to 12-μ) T colonies consistently grew out in gar subcultures. T colonies of identical morphology were obtained from luid cultures that had been inoculated with tissue-phase organisms. Simi-arly, the organisms in artificial culture medium (fluid and solid) could be re-erted to tissue phase by back inoculation into cell cultures. Adapted T

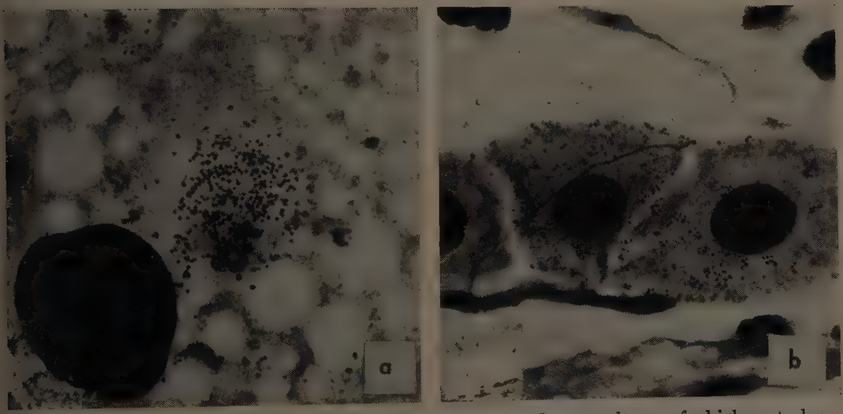

FIGURE 2. (*a*) T-strain PPLO in tissue phase. Monolayer culture of chick entodermal ells, fifth passage, 7 days postinoculation. Buffered azure-B eosin stain. ×1000. (*b*) 'arasitized epithelial cells from anterior urethra of a nongonococcal urethritis patient, illus-rating monomorphic, coccoid elements in the cytoplasm of the cells. Urethral scraping. iemsa stain. ×1000.

strains henceforth were cultivated in artificial fluid medium by subculture at 48- to 72-hour intervals. Adapted T strains thus maintained were later capable of rapid multiplication in HeLa cells[13] in which progressive cytopathogenic changes were induced. Adapted T strains were also capable of hemagglutinating chicken red cells to titers of 1:64 or more. The organisms were easily filterable through Selas filter candles of 03 porosity.

In conclusion, I have described some of the techniques employed in the recovery and propagation of T-strain PPLO, indicated their general characteristics, and have discussed some of the problems encountered in the propagation of freshly isolated strains.

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)IFFERENTIATION OF AVIAN PPLO AND BACTERIAL L FORMS*

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The history of the association of pleuropneumonialike organisms (PPLO) ith chronic respiratory disease (CRD) has been outlined by Adler *et al.*[1] It ates back to the findings of Nelson,[2] when he described a chronic coryza caused y an agent he termed coccobacilliform bodies. However, the significance f this association was not recognized. Later, a similar report by Markham nd Wong[3] initiated extensive studies on this problem. When it was found hat PPLO would produce a CRD-like infection,[3] isolation of the organism was sed in many laboratories as a method of diagnosis. Air-sac tracheal cultures ere prepared and examined for the presence of typical PPLO colonies.[4] These ere barely visible to the naked eye, but under 30 to 100 magnifications their enters appeared dense and were surrounded by a thinner outer portion, giving hem a characteristic "fried-egg" appearance.[5]

In the first attempts to cultivate avian PPLO at this laboratory various media ere used. However, PPLO were isolated from relatively few of the cases howing clinical lesions of CRD. Taylor *et al.*[6] had shown a marked difference n the ability of certain media to support growth. In their studies, the modiied technique of Grumbles *et al.*[7] and the method of Adler *et al.*[1] were considrably better than the other methods employed. Since both of these were ncluded in my studies, the reason for the relatively small number of isolations ould not be explained. It was assumed that the organisms were present, but hat some portion of the procedure was at fault. In order to evaluate the various nedia and techniques, cultures of avian PPLO were obtained from various aboratories. Bacteria were observed in many of the tubes that had been noculated with these avian PPLO cultures. The frequency of the appearance f bacteria was too great to be accounted for as contamination. Since no acterial inhibitors had been employed in any of the media, it appeared that eversions of the PPLO to bacteria may have been occurring in my cultures. AcKay and Taylor[8] previously had reported the reversion of cultures designated s PPLO to an organism resembling *Hemophilus gallinarum.*

If reversions were taking place, my cultures could not be classified as avian PLO, but would be L forms of some type of bacteria. Three aspects of this eneral problem are reported here. First, the frequency of bacteria obtained nder different culturing methods is reported. Second, a procedure is given or the differentiation of a bacterium as either a reversion from an L form or as contaminant. Finally, a discussion of the possible role of PPLO and bacerial L forms and their significance in CRD is presented.

MATERIALS AND METHODS

Media

Culture media were either Difco beef-heart infusion broth or agar. The roth was enriched by the addition of 1 per cent proteose peptone No 3, 1 per cent yeast hydrolyzate, and 1.5 per cent Difco PPLO-serum fraction. The agar was enriched with the same materials except that 1 per cent yeast extract was substituted for the yeast hydrolyzate. Sterilization was at 121° C. for 15 min. Serum fraction was added when the broth tubes or agar plates were prepared for use. All media were incubated for 48 hours at 37° C. and checked for sterility.

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One series of cultures was carried out in embryonated chicken eggs obtained from a flock of single-comb white Leghorns at the Pennsylvania State University poultry plant. Cultural or serologic tests for PPLO infection were not attempted on these birds. However, there has never been an outbreak of CRD in any of the chickens at the University poultry farm.

Methods of Culturing

Six different methods of cultivation were used to determine their effect on the frequency of the appearance of bacteria. Whenever bacterial growth was observed, slides were prepared and the morphology and Gram-staining reaction determined. No further tests for the identification of the individual bacteria were attempted.

Broth slants. Screw-cap test tubes containing 4.0 ml. of broth were incubated horizontally to increase the surface area of the medium. Four tubes were inoculated for each of 30 strains. The original inoculum was 0.3 ml., and this amount was transferred each Monday and Thursday for a total of 20 passages. All tubes were examined at 24-hour intervals following inoculation. When increased turbidity was observed, smears were prepared. If no bacteria were found on microscopic examination, passage was continued.

Agar-broth tubes. Agar slants were prepared using 4 ml. of agar in screw-capped tubes. To each tube was added 2 ml. of broth. Inoculation was made using 0.2 ml. of broth culture dropped onto the upper portion of the agar slant with the tubes in an upright position. The material thus ran down the slant into the broth serving as an inoculum for both media. Twenty-seven strains were transferred every Tuesday and Friday for a total of 16 passages.

Agar-broth flasks. The bottoms of 200-ml. Erlenmeyer flasks were covered with 30 ml. of agar which was allowed to solidify, and 30 ml. of broth was then added. Following 48-hour incubation for a sterility check, three flasks were inoculated with each of 12 strains. The volume of inoculum was 2.0 ml. After 7 days' incubation all cultures not showing bacterial growth were transferred to second flasks of the same type. These were incubated 7 days, after which time the experiment was terminated.

Daily transfers. Every 24 hours 5 ml. of broth culture was transferred to 50 ml. of broth in a 200-ml. Erlenmeyer flask. This was done for a total of 14 passages. Thirteen series of passages consisting of duplicate cultures of 5 strains and single cultures of 3 strains were made. When bacterial growth was observed, the morphology and Gram-staining characteristics were determined.

Increasing volumes. The total volume of culture was added to increasing volumes of media. The original inoculation of 2 ml. was made into 50 ml. o broth. After 48 hours at 37° C. the entire amount of 52 ml. was added to a flask containing 250 ml. of broth. This was incubated 4 to 5 days and th ntire amount (302 ml.) was then added to another flask containing 1000 ml. f no bacterial growth was evident after 7 days' incubation, this mixture (1302 nl.) was added to a final 2000 ml., making a total volume of 3302 ml. Samples vere checked for the presence of PPLO at the time of each transfer and again fter the final mixture had been incubated 7 days. A total of 48 cultures was arried through this procedure. They represented 2 or more cultures from ach of 22 different strains of avian PPLO.

Egg passage. Three cultures from each of 24 strains were passed 10 times n embryonated chicken eggs. Passage was by the yolk-sac route using 5-day-ld embryos. A sample of yolk was removed from each egg just prior to inculation. This yolk material was cultured in broth for 72 hours and was then ransferred to agar. The agar plates were examined after 24 and 72 hours for he presence of bacterial or PPLO-type colonies. If colonies of either type vere found, all materials subsequently originating from that egg were discarded. wo eggs were inoculated at each passage for each culture. If both eggs from given inoculum were alive after 114 hours' incubation, yolk was harvested rom one of the embryos picked at random and passed into 2 more embryos. f only one embryo remained alive at 114 hours, yolk material from it was used or passage into 2 embryos. Yolk was harvested from all embryos that died etween 18 and 114 hours' incubation and cultured in broth. This was coninued for 10 passages or until both embryos were found dead when the eggs vere candled on or before 114 hours' incubation. Passage was made using 0.2 nl. of yolk material that had been mixed with an equal volume of broth. All ggs, both alive and dead, were cultured at each passage in broth and then on gar, as described above. If both embryos were dead and only PPLO-type olonies were isolated, passage was terminated.

Routine passage of stock cultures. When a given group of cultures were used n a series of experiments they were transferred routinely at 5-day intervals. This allowed the use of fresh, actively growing cultures for all inoculations. ransfers were carried out using 0.1 ml. inoculum into 3 ml. of broth. These vere similar to the broth slant tubes, but were incubated in an upright position. No inhibitors were used, and cultures were checked at each passage by inoculaion onto agar.

Results and Discussion

The frequency of the recovery of bacteria when the 6 methods of culturing vere used is shown in TABLE 1. Bacteria were recovered from 152, or 48 per ent, of the 316 cultures tested. The agar-broth tube method gave the lowest requency, only 10 of the 27, or 37 per cent, showing bacteria. All others were elatively constant except the increasing-volume method, where 36 of the 48 ultures, or 75 per cent, yielded bacteria. Approximately 12 per cent of the ultures were lost during passage. If no growth was evident on the agar check lates of 2 successive passages, the culture was discarded. PPLO were still resent in 129, or 40 per cent, of the cultures after the final passages. This ncluded 42 cultures from the egg-passage group. Only 4 of these went through he full 10 passages in eggs. The other 38 caused embryo mortality without he presence of bacteria. They were designated as pathogenic for embryos, and passage was terminated, when both eggs of a given inoculation died before 114 hours and only PPLO were recovered.

Fifty per cent or more of the individual strains yielded bacteria by all methods except the agar-broth tubes. The number of cultures per strain giving bacteria varied from a low of 1 of 4 to as many as 12 out of 15. Three strains were used in the broth-slant group only; 1 of the 4 cultures of each yielded bacteria. All other strains were used for 2 or more culturing methods, and bacteria were recovered from at least 2 cultures of each strain. There was no significant difference between strains and, in most instances, bacteria were recovered from one or more cultures of each strain for each culturing method.

TABLE 1

FREQUENCY OF RECOVERY OF BACTERIA FROM AVIAN PPLO CULTURED BY SIX DIFFERENT METHODS

Culture methods	No. strains tested	Total No. cultures	No. of passages	Strains giving bacteria		Cultures giving bacteria		Cultures lost in passage	No. cultures PPLO retained
				No.	Percentages	No.	Percentages		
Broth slant	30	120	20	24	80	54	45	18	48
Agar-broth tube	27	27	16	10	37	10	37	6	11
Agar-broth flask	12	36	2	8	67	15	42	6	15
Daily transfer	8	13	14	7	88	7	54	3	3
Increasing volume	22	48	—	20	91	36	75	3	9
Egg passage	24	72	10	12	50	30*	42	1	42†
Totals		316				152	48	37 (12%)	129 (40%)

* Total of 99 isolations of bacteria.
† Thirty-eight of the 42 caused embryo deaths.

TABLE 2

GRAM STAINING AND MORPHOLOGY OF BACTERIA RECOVERED FROM AVIAN PPLO CULTURES

Culturing method	Cocci		Rods		Total No.
	Gram +	Gram −	Gram +	Gram −	
Broth slants	50	0	2	2	54
Agar-broth slants	6	0	3	1	10
Agar-broth flasks	8	0	5	2	15
Daily transfer	7	0	0	0	7
Increasing volume	34	0	2	0	36
Egg passage	75	0	15	9	99
Totals	180 81.4%	0 0	27 12.2%	14 6.4%	221

A Gram-stained smear was made of each bacterial culture; from this the morphology and staining reaction were determined. No further identification was attempted. Smears from 221 cultures were examined (TABLE 2). Gram-positive cocci were present in 180, or 81.4 per cent, of the cultures. Usually they were arranged singly, in pairs, or in clusters similar to those of *Micrococcus*. No Gram-negative cocci were found.

Smears of the other 41 cultures exhibited rod-shaped organisms of varying size and staining characteristics. Twenty-seven of these contained only large Gram-positive rods. The predominant organism in the other 14 cultures was a small thin Gram-negative rod. However, many of these also contained Gram-variable, highly pleomorphic forms that could not be identified.

The recovery of bacteria from approximately one half of the cultures indicated that these so-called avian PPLO were apparently L forms that were reverting to their bacterial form. Also, the different methods of culturing greatly influenced the appearance of the bacteria. Routine passages of 56 stock cultures in broth were checked for the presence of bacteria. After 10 transfers, only 2 cultures had exhibited bacterial growth. This is less than 4 per cent, as compared to the 37 to 75 per cent by the other culturing methods.

The frequent recovery of bacteria is highly indicative of the reversion of many of these cultures to their bacterial form. However, some of the bacteria recovered may be due to contamination. If detailed studies on L forms are to be conducted and their relationship to CRD determined, a method for the differentiation of true reversion and contamination is essential.

The frequent recovery of bacteria from cultures of avian PPLO indicated that one of two things had occurred. Either the PPLO were actually L forms and had reverted to their bacterial form, or an exceptionally large number of cultures became contaminated. The source of these bacteria can be determined now by a method developed for the identification of avian bacterial L forms.[9] When bacterial growth occurs in a PPLO culture, transfer is made onto 2 agar plates, 1 of which contains the bacterial inhibitors, penicillin and thallium acetate. The plate containing inhibitors is examined for the presence of PPLO-type colonies and, if none is found, the bacterium is considered a contaminant and discarded. However, when PPLO-type colonies are present, an isolated bacterial colony is picked from the plate without inhibitors and placed in broth. After 24 hours' incubation, the culture is again transferred to both kinds of agar. When only PPLO-type colonies develop on the agar containing inhibitors and only bacterial colonies on the plain agar, the bacterium is considered as having resulted from reversion, and the original culture is then classified as an L form.

The method of identification was developed after the trials with the 6 methods of culturing had been completed. After the morphologic and staining characteristics of the cultures had been determined, most of the bacteria were discarded. The bacteria obtained from the egg passages had been stored and are being carried through the identification procedures. This will allow a more accurate evaluation of the techniques employed and a determination of the frequency of reversion.

Detailed studies are being carried out using 14 selected cultures. Ten of these cultures are of the typical PPLO type described previously. They failed to produce any lesions following inoculation into chicks or turkey poults, and are classified as nonpathogenic. The other 4 cultures are classified as pathogens since they produce marked lesions following inoculation of both chicks and turkey poults. The colonies of these strains are much smaller than the colonies formed by the nonpathogens. Also, they grow considerably slower, requiring 4 to 6 days for growth to become evident on agar as compared to 24 to 48 hours with the nonpathogens. The six culturing methods described above as well as other techniques are being reevaluated. All bacteria derived from these procedures will be checked by the identification procedure.

Although the studies on these 14 selected strains are not advanced sufficiently to warrant reporting, one significant observation should be mentioned. In no instance has there been evidence of the reversion of any of the pathogenic strains. This is an additional marked difference between these pathogenic strains and the nonpathogenic strains. Adler *et al.*[1] showed that strains of avian PPLO differed in their serological reactions, rate of growth, flocculation with ammonium sulfate, and pathogenicity. Their ability to revert to bacteria now can be added to the list.

On the basis of these differences, a working hypothesis is proposed. The so-called avian PPLO are divided into two groups. First, the pathogenic strains are considered as the true PPLO or *Mycoplasma*. These are the ones that apparently play an important part in the etiology of CRD. The second group or nonpathogens are considered as a completely different type of organism. Since bacteria have been derived from all strains tested by at least one or more methods of culturing, they cannot be included as PPLO and must be classified as bacterial L forms. The significance of these L forms or of the bacteria from which they originate with respect to CRD is not understood. However, they are frequently isolated from birds suffering from CRD and have complicated the entire picture greatly. Either their elimination as a factor in CRD or the determination of their part in this disease complex is essential.

Summary

Avian PPLO were cultured by 6 different methods, and the frequency of the recovery of bacteria was determined. Bacteria became evident in from 37 to 75 per cent of the cultures transferred by these methods, as compared to less than 4 per cent when routine passage procedures were used. The bacteria recovered were predominantly Gram-positive cocci.

A procedure for identifying reverted bacteria is described. This is carried out using inhibitor and noninhibitor agar plates. The cultures showing actual reversion to the bacterial form then must be classified as bacterial L forms.

As a working hypothesis, avian PPLO are divided into two groups according to several distinct differences. The pathogenic strains are classified as true PPLO or *Mycoplasma*. The nonpathogenic strains revert to bacteria and are classified as L forms.

The role of L forms in the CRD complex must be determined before a complete understanding of this disease can be obtained.

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DERIVATION OF GRAM-POSITIVE COCCI FROM PLEUROPNEUMONIALIKE ORGANISMS*

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Three schools of thought are evident concerning the relationship of the pleuropneumonialike organisms (PPLO) to L forms of bacteria. First, it has been reported that PPLO had their origin separate from the L forms, and that the two groups are not related (Edward, 1950, 1953; Edward and Fitzgerald, 1951; Edward and Freundt, 1956; Bartmann and Höpken, 1955). The second school of thought was presented by Tulasne (1951) and Tulasne and Bringmann (1952), who agree that PPLO should be classified separately from the L forms but believe that PPLO were originally L forms of bacteria that have become fixed in that form. Finally, reports appear indicating that PPLO as such may not exist and that the organisms now designated as PPLO may be converted to bacterial forms under proper environmental conditions. This view is supported by Dienes and Weinberger (1951), who minimized the differences between L forms and PPLO, and by the reports of Minck (1953), McKay and Taylor (1954), Peoples *et al.* (1955), Wittler *et al.* (1956), and Smith *et al.* (1957), in which the derivation of bacterial forms from PPLO has been described.

In this paper evidence is presented to indicate a possibility that, under proper environmental conditions, most PPLO may be converted to a bacterial form.

Materials and Methods

Media. The four types of media used were designated as broth or agar where no serum component was used and as serum broth or serum agar where 10 per cent horse serum was incorporated.

Broth medium was made with Difco heart-infusion broth fortified with 1 per cent Difco yeast extract and 1 per cent Difco proteose peptone No. 3. In the experiments with the avian PPLO, no adjustment of the *p*H was made. In the other experiments, the *p*H was adjusted to 7.9 with NaOH prior to sterilization. The broth medium was tubed and autoclaved at 121° C. for 15 min. Serum broth was prepared by adding 10 per cent sterile horse serum to the tubes of broth medium. The *p*H of the unadjusted serum broth was 7.3. No change in the *p*H of the adjusted broth medium was apparent after 10 per cent horse serum was added. All tubes were incubated for at least 48 hours at 37° C. prior to use.

The agar medium contained the same ingredients except that Difco heart-infusion agar was used instead of the heart-infusion broth. The agar medium

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was prepared in 100-ml. amounts and autoclaved. For culturing the bacterial forms, no adjustment was made of the *p*H. To prepare serum-agar plates, the *p*H of the 100-ml. amount of agar medium was adjusted to a higher level prior to sterilization by adding the same amount of NaOH as was required to bring 100 ml. of broth medium to *p*H 7.9. The 100 ml. of agar medium was melted and cooled to 56° C. Ten milliliters of sterile horse serum were added, and the plates were poured.

All horse serum was sterilized by filtration through a Seitz filter and inactivated at 56° C. for 30 min. prior to use. At no time in these experiments or in the purification of the PPLO strains was any bacterial inhibitor used.

Organisms. Included in this study were 21 PPLO of avian sources as follows: Univ. of Pa., strains J, I; Va. Poly. Inst., BP-8, A-5; Ala. Poly. Inst., 147, 189, 5820, 5109; Univ. of Calif., Davis, A-36; N.Y. Vet. Coll., 85, 91-R, 41-R, 111-R; Univ. of R.I., CRD-Masi, CRD-Swift; San Gabriel, Calif., 2682 Bird No. 1, 2682 Bird No. 2, 2683 Bird No. 1; and isolated at Penna. State Univ., PSU-4, PSU-5, and PSU-7.

Also included were 3 human strains: Mass. Gen. Hosp., 4330; Univ. of Pa., O7, 39; three canine strains: Walter Reed Army Inst. of Res., PG-13, PG-14, PG-15; and a saprophytic strain: Mass. Gen. Hosp., C-15.

The canine strains were originally isolated by D. G. ff. Edward, Wellcome Research Laboratories, Beckenham, Kent, England.

Purification of the PPLO strains. Each PPLO strain was purified by picking isolated colonies with an inoculating loop and streaking on serum agar through 5 successive transfers. Under a dissecting microscope, an isolated colony was rubbed with the loop until the dense central portion disappeared and the colony was then streaked to the new agar (or introduced into broth medium). Following 5 single-colony transfers, inoculation was made into 5 ml. of serum broth. After 48 hours' incubation, the serum-broth culture was distributed into small sterile test tubes in 0.5-ml. amounts and stored at −20° C. At this temperature all strains remained viable for at least 5 months, and strain J was still viable after 1½ years. All experiments were initiated from the frozen stock cultures.

Culture methods for derivation of bacterial forms. All media used for culturing avian strains were prepared without adjustment of the *p*H. One-tenth-milliliter amounts of 48-hour cultures of 21 strains were transferred to 2 ml. of broth (containing no serum component) in cotton-stoppered tubes 1.5 cm. o.d. All tubes were incubated in an upright position. After 48 hours' incubation at 37° C., transfer was made to tubes of the same size containing 1.5 ml. of serum broth. This transfer was designated as the first transfer. Passage was made by loop into the same type of tube containing 1.5 ml. of serum broth at 48-hour intervals until bacterial turbidity appeared in all cultures. The bacterial forms were passed on agar medium.

The bacterial form derived from strain J failed to grow on the agar medium. The bacterial form was derived a second time from this strain by the technique described in the next paragraph.

The human, canine, and saprophytic strains of PPLO were treated essentially the same as the avian PPLO except for the following modifications. The *p*H of the serum broth was 7.9, and screw-cap test tubes, 1.5 cm. o.d. × 13 cm.

deep and containing 1.5 ml. of serum broth, were used in passing the cultures. The screw-cap test tubes were incubated in an almost horizontal position to give maximum surface area and minimum depth. This method of incubation, with passage at 48 to 72 hours, will be referred to as the shallow-broth technique. As the bacterial forms appeared, they were passed on agar medium.

Effect of serum concentration on PPLO cultural stability. In using the shallow serum-broth technique it was found that, if bacterial forms did not appear in the earlier passages, they were not likely to appear in later transfers. The following experiments demonstrated this phenomenon.

Sets of 12 screw-cap test tubes containing 1.5 ml. of serum broth were inoculated from 48-hour cultures of PPLO strains PSU-5, J, and 4330, each tube receiving 1 drop from a 1 ml. pipette. This inoculation was designated as the first transfer, and passage by the shallow-broth technique was made at 48- to 72-hour intervals until bacterial forms appeared or until the experiments were discontinued.

In an effort to derive bacterial forms from stable cultures, the horse serum concentration was reduced gradually as the cultures were passed. PPLO strains PG-13, PG-14, and PG-15 also were included.

After a number of transfers (as indicated for the strains in the second column of TABLE 2) in serum broth containing 10 per cent horse serum and using the shallow-broth technique, transfers were made into broth containing 7 per cent horse serum. Two more passages were made with the horse-serum level at 7 per cent. The cultures were transferred to broth containing 6 per cent horse serum. This process was continued; at each third transfer the horse-serum concentration was reduced 1 percentage unit. With the cultures that survived through the 1 per cent horse-serum level, the process was continued with broth containing 0.5, 0.25, and 0.1 per cent horse serum. At the 1.0 and the 0.1 per cent levels, transfers were made into broth containing no horse serum. All transfers were made by loop. All cultures were incubated by the shallow-broth technique at 37° C. until turbidity was visible. If turbidity did not appear after 15 days' incubation, it was assumed that the culture failed to grow, and transfers were discontinued.

The final culture at each horse-serum level was transferred to serum agar (containing 10 per cent horse serum) to observe changes in the colonial morphology.

Results

Derivation of bacterial forms from avian PPLO. On the second transfer in broth 6 of the avian PPLO cultures showed bacterial turbidity; on the third transfer the remainder of the 21 cultures showed bacterial growth. Strain J, although having shown bacterial turbidity, failed to grow when transferred to agar medium. The other 20 strains of bacteria showed gray punctiform colonies, flat and ragged on the edge. FIGURE 1*a* shows colonies of this type, which were derived from the culture of strain I on the first transfer to agar medium. Single colonies were streaked to new agar medium through 6 transfers. As the bacteria were transferred the colonies gradually became more mucoid in appearance and white in color. The Gram morphology of the

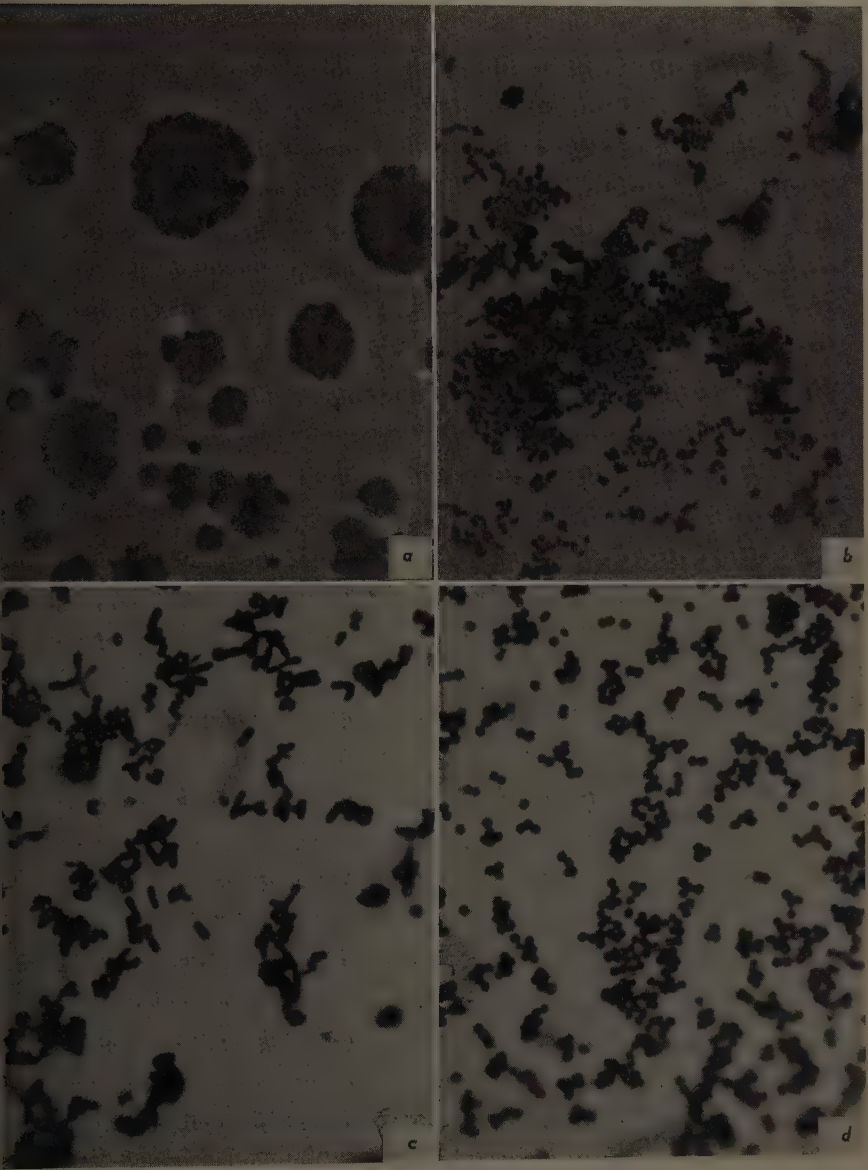

FIGURE 1. Bacteria derived from avian PPLO strain I. (*a*) Colonies on first transfer to gar medium from PPLO culture in which bacteria appeared. Unstained. Large colony in pper center about 0.3 mm. Low power. ×60. (*b*) From colonies shown in *a*. Gram-egative forms predominate, with occasional Gram-positive forms present. ×1000. (*c*) After continued transfer on agar medium until colonies were whitish and slightly mucoid in ppearance. Gram-negative forms absent. ×1000. (*d*) After continued transfer on agar edium until colonies were white, mucoid, and circular (about 1 mm.). Cells about 1 μ n diameter. Culture was identified as *Micrococcus*. ×1000.

bacterial forms also underwent considerable change. Immediately on derivation from the PPLO culture the bacterial cells were Gram-negative and rod-shaped. As transfers on agar were made the cells became Gram-positive, larger in size, and showed a high degree of pleomorphism. Finally, the individual organisms took on the characteristics of *Micrococcus*. FIGURE 1*b*, *c*, and *d* shows these stages in the development of the bacterium derived from strain I.

Seventeen of the 20 bacterial cultures were determined to be members of the genus *Micrococcus* on the basis of the results of the following tests: colonial morphology on nutrient agar—circular, white (except for the culture from strain I, which was yellow), convex, mucoid colonies about 1 mm. in diameter; Gram morphology—Gram-positive cocci about 1 μ in diameter as single cells, pairs, and grapelike clusters; and abundant mucoid growth on *Staphylococcus* 110 agar (Chapman, 1948).

These 17 strains were also catalase-positive and failed to grow at 10° C. and on heart-infusion agar containing 0.1 per cent methylene blue. The final *p*H of glucose-broth cultures (Difco phenol-red broth containing 0.5 per cent dextrose) fell in the range of 4.40 to 4.75. These last tests were made to assure ourselves that none of the strains were members of the fecal *Streptococcus* group.

The bacteria derived from strains PSU-5 and PSU-7 were identified as *Streptococcus faecalis* var. *liquefaciens*. These bacterial strains also showed the developmental stages described above. FIGURE 2*a*, *b*, and *c* shows the developmental stages of the bacteria derived from strain PSU-5.

Their identification as *Str. faecalis* var. *liquefaciens* was based on the following results: punctiform blue-gray colonies on nutrient agar; good growth at 10° C. and 45° C.; good growth on heart-infusion agar containing 0.1 per cent methylene blue; negative for the production of catalase; final *p*H in glucose-broth cultures, 4.10 and 4.03, respectively; rapid liquefaction of gelatin; and failure to show hemolytic ability on blood agar.

The colonies of one bacterial strain, from BP-8, retained the gray and flat punctiform characteristics on completion of the 6 transfers on agar. This strain showed no fermentative ability, and required 48 to 72 hours to appear. The Gram stain showed Gram-positive coccoidal forms. This bacterial strain was catalase-negative and failed to grow on blood agar or gelatin. It is interesting to note, however, that it showed fair growth at 48 hours on *Staphylococcus* 110 agar. At the time these tests were performed this strain was tentatively identified as a diphtheroid. After two months' storage at −20° C., it was found that all stored tubes of this strain produced typical cultures of *Micrococcus*.

In addition, all the bacterial cultures were submitted to the following tests: action on blood agar, ability to coagulate rabbit serum, production of nitrites from nitrates, production of indole, action on gelatin, action on litmus milk, and ability to ferment 12 carbohydrates.

On the basis of the results of all the tests, it was found that only 2 of the cultures of *Micrococcus* were identical. Each of the others showed characteristics that separated it from the rest of the group. Thus, 18 distinct bacterial strains were found in the 20 cultures.

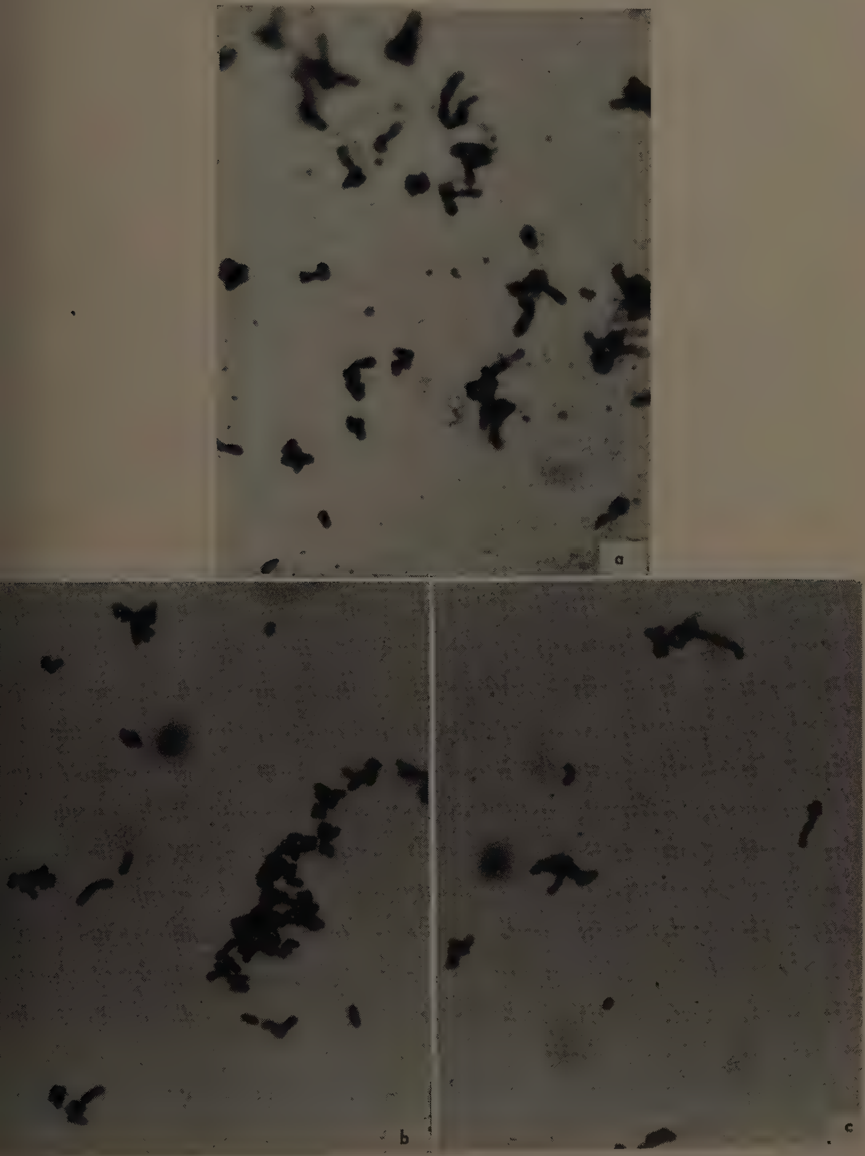

FIGURE 2. Bacteria derived from avian PPLO strain PSU-5. Gram stains. Oil immersion. ×1000. (*a*) After 5 transfers on agar medium. (*b*) After 12 transfers on agar medium. (*c*) After 15 transfers on agar medium. Cells about 0.8 μ. Culture was identified as *Str. liquefaciens*.

Bacterial forms from human, canine, and saprophytic PPLO. Bacterial forms were derived on transfers by the shallow-broth technique as follows:

Strain 4330	third transfer
Strain O7	first transfer
Strain 39	second transfer
Strain PG-13	seventh transfer
Strain PG-14	thirteenth transfer
Strain PG-15	eighth transfer
Strain C-15	eighth transfer

It was noted in these experiments that changes in turbidity of the PPLO culture occurred one or two transfers prior to the appearance of the bacterial forms. The PPLO culture showed decidedly heavier turbidity, even producing a whitish sediment in the bottom of the tube. Smears of the heavily turbid cultures showed no bacterial forms and, when transferred to serum agar, only PPLO colonies were produced.

The bacterial forms derived from these strains passed through the stages of development described previously. FIGURE 3*a*, *b*, *c*, and *d* shows stages of the bacteria from strain 39.

The bacterial forms from these 7 PPLO were identified as strains of *Micrococcus* on the basis of the identifying tests described previously.

Stable PPLO cultures. Stable cultures were demonstrated with the three strains used in this experiment. TABLE 1 shows that 10 cultures of strain PSU-5 produced bacterial forms. Two cultures, however, were transferred through 57 passages without producing any bacteria. The same phenomenon was demonstrated by 6 cultures of strain J and by 7 cultures of strain 4330.

TABLE 1 indicates that a large majority of the bacterial forms passed through the developmental stages described previously. Some of these bacterial cultures, however, apparently became fixed at the stages represented by FIGURES 1*c*, 2*b*, and 3*b*. Passage through as many as 20 transfers on heart-infusion agar failed to produce the typical *Micrococcus*. The colonies were punctiform, raised, whitish in color, and slightly mucoid. On the other hand, 2 cultures from strain PSU-5, 3 cultures from strain J, and the 2 cultures from strain 4330 passed through the intermediate stages within 12 passages and presented bacterial cultures of *Micrococcus* (from J and 4330) and *Str. faecalis* var. *liquefaciens* (from PSU-5).

Gradual reduction of horse serum. The results of these experiments are shown in TABLE 2. A general pattern was exhibited by strains PSU-5, J, and 4330. With the horse-serum concentration in the range from 7 per cent through 4 per cent, the PPLO cultures showed slight turbidity in 3 to 5 days. In the lower levels, however, decidedly heavier turbidity was exhibited. In the range from 3 to 1 per cent horse serum, turbidity was clearly evident at 24 hours, and by 48 to 72 hours a white sediment could be found in the bottom of the tube. On longer incubation of 5 to 7 days, these cultures showed small white particles clearly visible to the naked eye. Smears made from the heavily turbid cultures and stained with crystal violet showed no bacterial forms. The filamentous aggregates shown in FIGURE 4*a* were abundant.

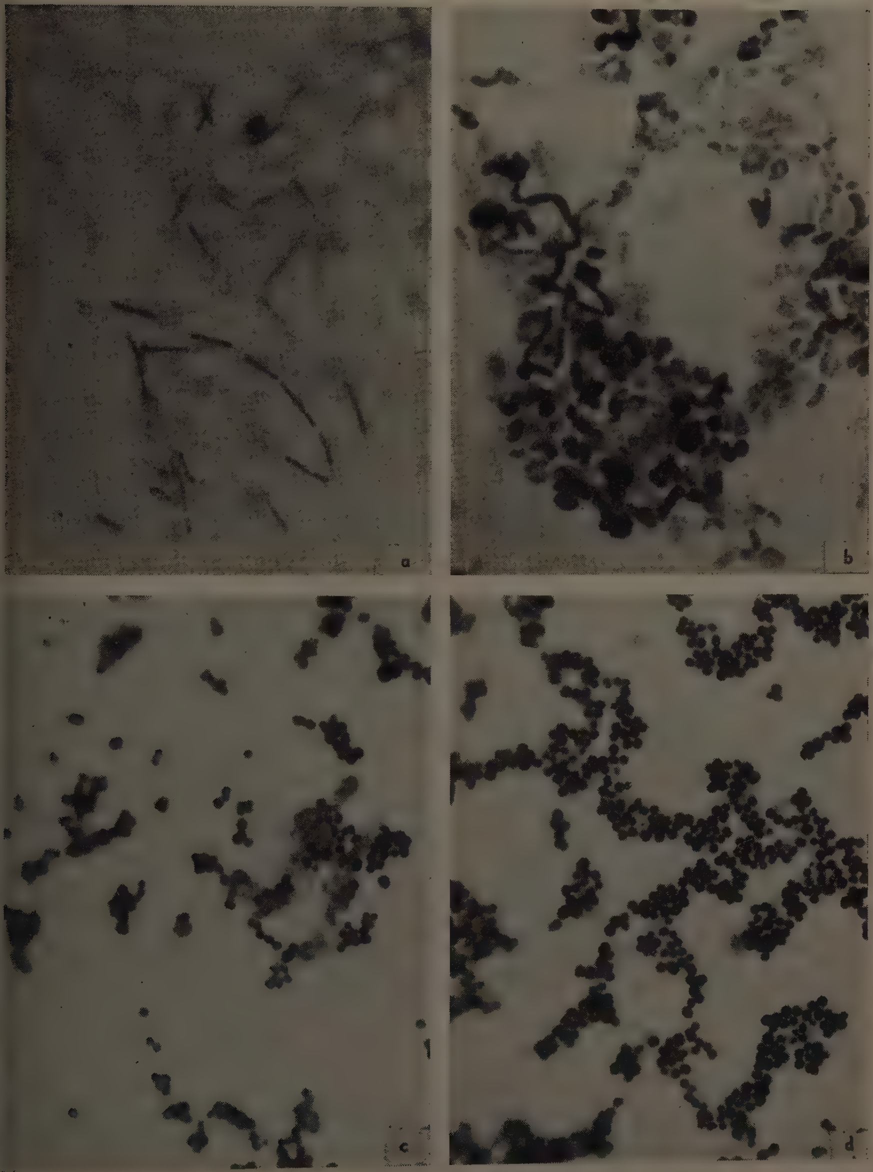

FIGURE 3. Bacteria derived from human PPLO strain 39. Gram stains. Oil immersion. ×1000. (*a*) Smear from broth (PPLO) culture in which bacteria appeared. Entire culture was Gram-negative. (*b*) After 2 transfers on agar medium. Dark forms are Gram-positive. Lighter forms are Gram-negative. (*c*) After 6 transfers on agar medium. Some Gram-negative forms still present. (*d*) After 7 transfers on agar medium. Cells about 1 μ. Culture was identified as *Micrococcus*.

Changes in the colonial morphology became apparent as changes in turbidity occurred. With the horse-serum concentration in the range of from 7 to 5 per cent, turbidity was typical of PPLO. When transferred to serum agar containing 10 per cent horse serum, these cultures showed PPLO colonies of normal appearance (FIGURE 4*b*). In the cultures containing lower concentrations of horse serum, the turbidity became coarser and decidedly heavier. When transferred to serum agar, the colonies were larger, less regular in outline, and coarse

TABLE 1

RESULTS OF EXPERIMENT IN WHICH 12 TUBES EACH OF SERUM BROTH WERE INOCULATED WITH THREE PPLO STRAINS AND CULTURED BY THE SHALLOW-BROTH TECHNIQUE

PPLO strain	Passage at which bacterial forms appeared	Intermediate bacterial forms demonstrated	Transfers discontinued
PSU-5	2 cultures, passage 1	yes	2 cultures at passage 57
	1 culture, passage 7	yes	
	1 culture, passage 10	yes	
	1 culture, passage 12	yes	
	1 culture, passage 18	yes	
	3 cultures, passage 25	yes	
	1 culture, passage 27	yes	
J	2 cultures, passage 5	yes	6 cultures at passage 47
	2 cultures, passage 8	yes	
	2 cultures, passage 10	yes	
4330	2 cultures, passage 7	yes, no	7 cultures at passage 54
	1 culture, passage 9	no	
	2 cultures, passage 17	yes, no	

TABLE 2

GROWTH OF STABLE PPLO IN SERUM BROTH IN WHICH THE CONCENTRATION OF HORSE SERUM (HS) WAS GRADUALLY REDUCED

Strain	Transfers prior to reduction of horse serum	Number of cultures tested	Bacterial forms derived	Level of HS which failed to support growth
PSU-5	47	2	0	1%
J*	27	6	2 in 3% HS	0.5%
4330*	31	7	1 in 4% HS 1 in 3% HS 1 in 2% HS 1 in 1% HS	Good growth through 0.1% HS. Transfers discontinued.
PG-13	6	6	1 in 7% HS	4%
PG-14	6	6	1 in 5% HS	3%
PG-15	6	6	0	4%

* Transfers from cultures at 1 and 0.1 per cent horse-serum levels to broth containing no horse serum failed to grow.

in surface texture. In these cultures numerous colonies were found that were 3 to 5 times larger than the normal colony and showed indistinct central portions (FIGURE 4*c* and *d*). The larger colonies were surface-growing and could be removed with an inoculating loop, although a thin plaque the shape of the colony remained adhering to the agar. When transfers were made from cultures of strain J at the 2 per cent horse-serum level to serum agar, the entire culture was composed of the surface-growing colonies with no discernible central portions (FIGURE 4*e*).

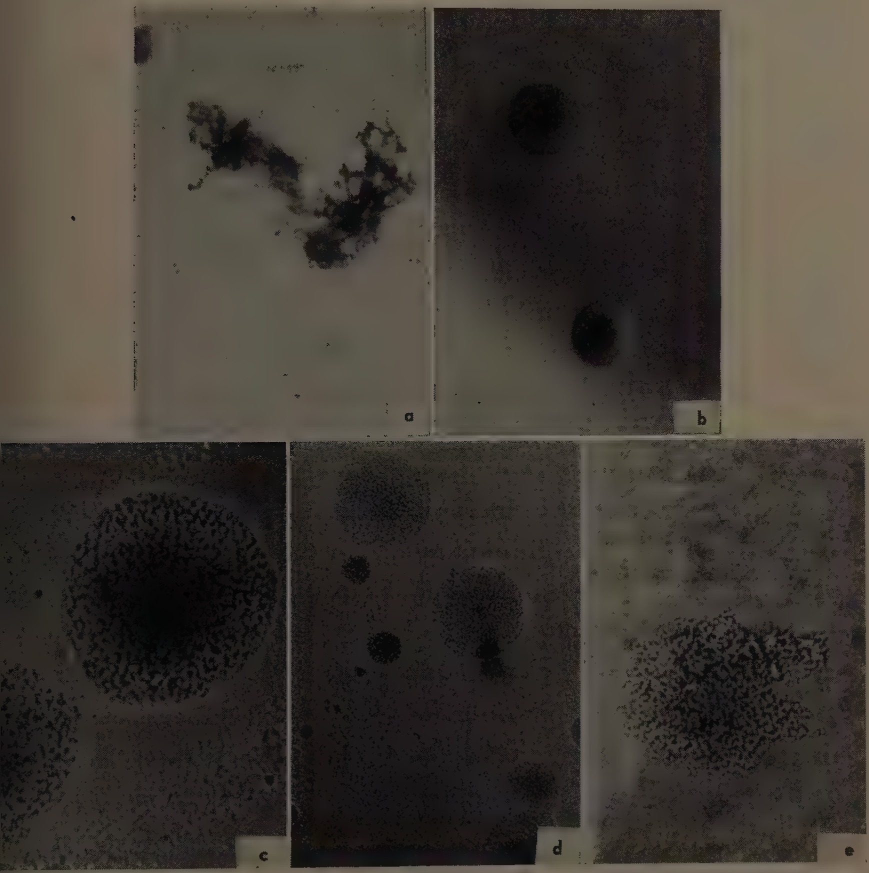

FIGURE 4. (*a*) Smear from culture of PPLO strain J with horse serum at 1 per cent level and showing heavy PPLO turbidity. Crystal violet. Oil immersion. Portion of aggregate to lower right, about 8 × 16 μ. ×1000. (*b*, *c*, *d*, and *e*) PPLO colonies cultured on serum agar with horse serum at 10 per cent. Unstained. Low power. ×100. (*b*) Normal-appearing colonies of strain J. Colonies about 0.1 mm. (*c*) Strain PG-15 transferred from broth culture with horse-serum level at 5 per cent. (*d*) Strain 4330 transferred from broth culture with horse-serum level at 0.25 per cent. (*e*) Strain J transferred from broth culture with horse-serum level at 2 per cent.

Discussion

Three possibilities can be considered as sources of the bacterial forms. First, the bacteria may have entered the tubes as contaminants. Second, the bacteria liberated by the techniques used may have been present in the cultures in a latent state. The third source would be the conversion of the PPLO to the bacterial form.

Evidence against the possibility of contamination was presented in the experiment with the avian PPLO. Twenty strains of bacteria were obtained in transferring 21 PPLO cultures 3 times. In addition, it was found that 18 characteristically different bacteria were represented in the 20 strains. The frequency with which the bacteria appeared and the wide variety of strains obtained make random contamination highly improbable.

The purification method of streaking isolated PPLO colonies presented evidence against the second possibility, the liberation of inhibited bacteria. It would appear that strains of *Micrococcus* and *Str. faecalis* var. *liquefaciens* would grow more vigorously than PPLO. It would seem unlikely, therefore, that the vigorously growing bacteria would be held in a latent state within the PPLO colony or within the PPLO streaked from a single colony over the surface of the agar.

Evidence favoring the conversion of PPLO to bacterial forms was presented in the apparent enlargement of the PPLO as indicated by the increased turbidity just prior to the appearance of the bacteria and in the stages of development through which the bacterial forms passed. These phenomena indicated a transition of PPLO to bacterium.

Transition was indicated also in the experiments with reduced horse serum. Increased turbidity of the successive PPLO cultures indicated a possible increase of cell size. In addition, the colonies were considerably larger than the normal PPLO colony, were surface-growing and, in some cases, showed no discernible central portions. These colonies appeared to represent intermediate colonies having both PPLO and bacterial characteristics.

Summary

Techniques are described with which bacterial forms were derived from 21 avian, 3 human, 3 canine, and 1 saprophytic strain of PPLO. Two of the bacterial strains derived from avian PPLO were identified as *Str. faecalis* var. *liquefaciens*. The other 27 bacterial cultures were identified as strains of *Micrococcus*.

In one experiment bacterial forms were derived from 21 avian PPLO within 3 transfers in shallow-broth cultures. Within these cultures, 18 characteristically distinct strains of bacteria were obtained. These data are presented as evidence against contamination as the source of the bacteria.

It was observed that the PPLO culture became more turbid just prior to the appearance of the bacterial forms and that the bacteria then passed through stages of development until they finally attained the characteristics of *Micrococcus* or *Str. faecalis* var. *liquefaciens*. These phenomena are presented as evidence in favor of the conversion of PPLO to bacteria.

In transferring PPLO in shallow-broth cultures, it was found that, if bacteria

did not appear in early passages, they were not likely to be derived in later passages. Efforts were made to derive bacterial forms from these stable PPLO cultures by gradually reducing the concentration of horse serum. Bacterial forms were obtained from 4 of 6 PPLO. In addition, considerable change was observed in the PPLO cultures. Decidedly heavier turbidity was observed and, when transferred to agar medium, the colonies were surface-growing and much larger than normal PPLO colonies and, in some cases, showed no discernible central portion.

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GROWTH-CURVE STUDIES OF PLEUROPNEUMONIALIKE ORGANISMS*

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In 1932 Holmes and Pirie[1] attempted to construct growth curves of the bovine pleuropneumonia organism by correlating the degree of reduction of methylene blue with the amount of growth. Keller and Morton[2] pointed out the futility of turbidimetric measurements of whole cultures in the construction of growth curves of human strains of pleuropneumonialike organisms (PPLO). These workers determined a growth curve using a colony-count method in which the volume of inoculum was correlated to the surface area of a field of the microscope, and the average of 100 fields was used as the count.

The work on quantitative determinations of PPLO has been reviewed by Smith.[3] A study of the methods for determining PPLO growth was presented, and it was concluded that the following techniques were suitable for general laboratory use: (1) measurement of turbidity following concentration of the culture by centrifugation; (2) measurement of the colony diameter; and (3) colony count using 0.01 ml. of culture (or diluted culture) deposited on the surface of agar, incubated, and counted under low magnification.

In this study, growth curves of 2 human, 2 avian, and 1 saprophytic PPLO were determined using a colony-count method of assay. The method appears to be accurate and requires no complicated techniques or special equipment.

Materials and Methods

Growth curves were determined on the following strains of PPLO:

Strain No.	*Source*	*Obtained from*
PSU-5	Infected chicken embryo	Pennsylvania State University, University Park, Pa.
I	Trachea of chicken	University of Pennsylvania, Philadelphia, Pa.
39	Human urethra	University of Pennsylvania
4330	Female GU tract (human)	Massachusetts General Hospital, Boston, Mass.
C-15	Compost (saprophytic)	Massachusetts General Hospital

Media. Broth medium for growth-curve cultures was prepared with Difco heart-infusion broth, to which 1.0 per cent Difco yeast extract and 1.0 per cent Difco proteose peptone No. 3 were added. The yeast extract was omitted in some of the growth-curve cultures. The ingredients were dissolved in distilled water, and the pH was adjusted to 7.9 with NaOH. The medium was distributed into screw-cap vials 2.5 cm. o.d. × 10 cm. deep in 30-ml. amounts nd autoclaved at 121° C. for 15 min. Three milliliters of sterile horse serum vas added to each vial.

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Agar medium consisting of Difco heart-infusion agar with 1.0 per cent Difco east extract and 1.0 per cent Difco proteose peptone No. 3 was prepared in 00-ml. amounts. The same amount of NaOH was added as had been required to adjust 100 ml. of the broth medium to *p*H 7.9. After sterilization t 121° C. for 15 min., the medium was cooled to 56° C. Ten milliliters of terile horse serum was added, and the plates were poured, using Petri dishes f 60 mm. diameter. About 15 plates were obtained from each 100 ml. of agar nedium. The plates were allowed to remain overnight at room temperature o permit the surface of the agar to dry.

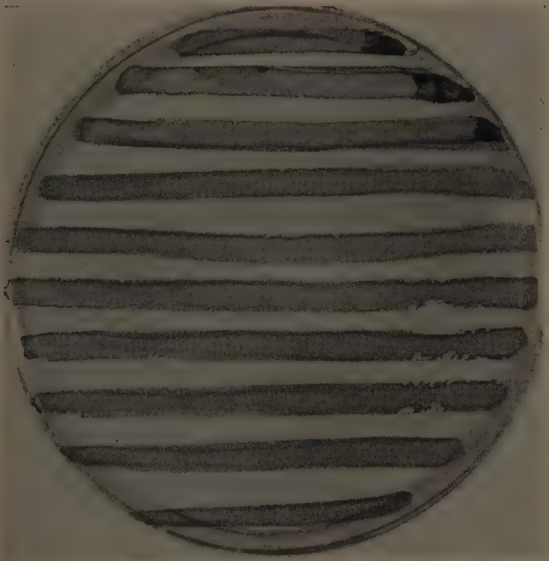

FIGURE 1. Underside of Petri dish lined off with transparent ink in preparation for countng colonies.

Growth-curve determination. The growth-curve culture vials, each containing 0 ml. of broth, were inoculated with a small loopful of 48-hour cultures of the PLO strains. After thoroughly mixing, a 0.5-ml. sample was removed as the ero-hour sample. The vial was incubated at 37° C., and 0.5-ml. samples were emoved after 3, 6, 8, 14, 24, and 36 hours, and at approximately 24-hour inervals thereafter. The 0.5-ml. samples were used to prepare serial tenfold ilutions in screw-cap test tubes containing 4.5 ml. of sterile 0.5 per cent soluion of Difco proteose peptone No. 3 in distilled water. One-tenth-milliliter mounts of each dilution were plated in duplicate. During the first 24 hours, .1-ml. amounts also were plated directly from the growth-curve culture and esignated as 10^{-1} dilutions. All plates were incubated for 72 hours at 37° C.

To count the colonies, parallel lines were drawn across the bottom section f the Petri dish (FIGURE 1) with a felt point pen using transparent glassnarking ink. With the lid removed, the colonies above the inked portions were clearly visible (FIGURE 2) under a dissecting microscope with ×30 magnification. Counts up to 1000 colonies per plate were made without difficulty.

Analysis of the logarithmic phase of growth. Assuming that binary fission was the mode of reproduction, the generation time for each strain was computed, using the numbers of organisms representing the first and last points on the logarithmic phase of growth as the initial and final concentrations of organisms. The equations presented by Oginsky and Umbreit[4] were used. Starting again with the number of organisms representing the first point of the logarithmic growth phase and assuming that this population doubled at each generation time interval, the population was calculated for each time point where there were corresponding experimental data. These data were compared to the values calculated under the assumption that binary fission occurred. Strain C-15 showed only two points on the logarithmic phase. This comparison, therefore, could not be made and only the generation time was determined for this strain.

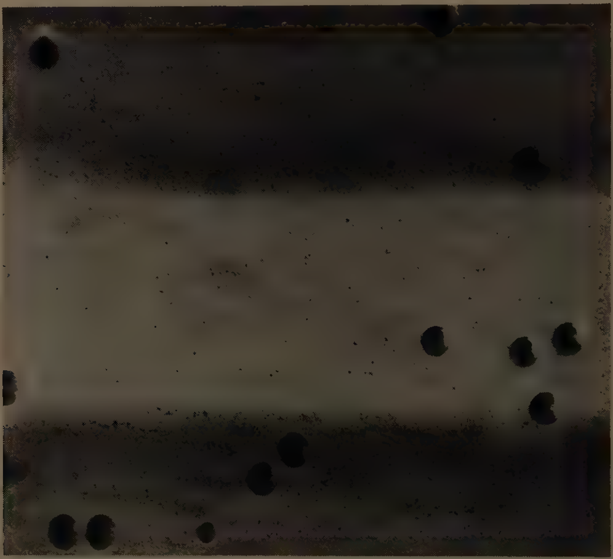

FIGURE 2. Field of dissecting microscope, ×40, showing colonies of PPLO over the lined areas and in the space between the lined areas. Unstained. Colonies approximately 0.1 mm.

By the same method as described for the growth-curve determinations, a culture of strain I was initiated, and samples were removed and assayed at each hour from 14 through 23 hours of growth (FIGURE 3). Using the 14-hour assay as the initial population and assuming that the population doubled at the generation time interval (1.3 hours), the populations were computed as shown in TABLE 2. The computed populations were then compared with the populations obtained by the plate-count assays for the corresponding times.

Results and Discussion

The growth curves of the PPLO strains are shown in FIGURES 4 through 8. Although there is variation in the shapes of the growth curves, all showed haracteristics of normal bacterial growth curves. The lag phases varied rom 2 to 13 hours, but all strains showed a straight line logarithmic phase of rowth.

Strains I, PSU-5, and 39 (FIGURES 5, 6, and 7) apparently produced autotoxic ubstances during growth. The presence of the yeast extract caused more

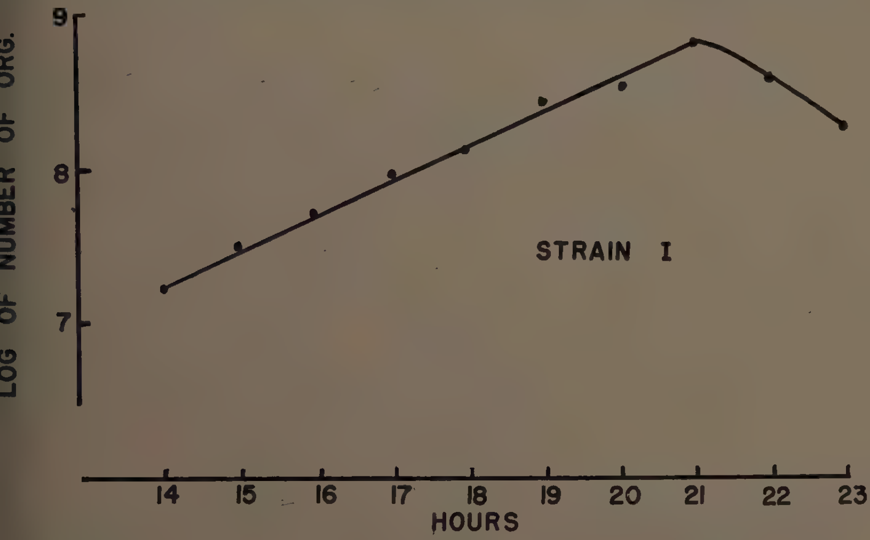

FIGURE 3. Strain I. Plots of assays made hourly from the fourteenth through the twenty-hird hours of growth.

TABLE 1

COMPARISON OF POPULATIONS OF PPLO DETERMINED BY PLATE-COUNT ASSAY DURING THE LOGARITHMIC PHASE OF GROWTH WITH POPULATIONS COMPUTED UNDER THE ASSUMPTION THAT BINARY FISSION WAS THE MODE OF REPRODUCTION

Strain	Generation time (hours)	Hours of growth	Plate-count populations	Computed populations
39	2.410	2	1.25×10^3	
		6	3.97×10^3	4.15×10^3
		8	9.10×10^3	7.45×10^3
		13	3.84×10^4	3.13×10^4
		25	9.25×10^5	9.88×10^5
I	0.962	3	6.45×10^2	
		6	3.55×10^3	5.77×10^3
		10	5.42×10^4	1.05×10^5
		14	1.78×10^6	1.32×10^6
PSU-5	0.810	3	4.50×10	
		6	5.70×10^2	5.65×10^2
		10	9.50×10^3	1.15×10^4
		14	5.59×10^5	5.73×10^5
4330	1.655	10	3.85×10^3	
		14	1.55×10^4	2.19×10^4
		25	4.15×10^6	2.00×10^6
		32	3.88×10^7	4.10×10^7
C-15	1.760			

rapid growth with these three strains, but there was also a more rapid decline of viable organisms when the yeast extract was present.

Saprophytic PPLO are not dependent for growth on serum component in the medium. Strain C-15, a saprophyte, was maintained in this laboratory on medium containing 10 per cent horse serum. No efforts were made to adapt the strain to medium containing no serum prior to determination of the growth

TABLE 2

COLONY COUNTS AND POPULATIONS OF STRAIN I DETERMINED DURING THE LOGARITHMIC PHASE OF GROWTH AS COMPARED WITH POPULATIONS COMPUTED ASSUMING BINARY FISSION AS THE MODE OF REPRODUCTION

Hour	Duplicate plate counts at dilutions:				Plate-count populations	Computed populations
	10^{-5}		10^{-6}			
14	147	164	10	18	1.55×10^7	
15	332	284	36	32	3.08×10^7	2.74×10^7
16	511	490	46	58	5.00×10^7	4.77×10^7
17	T*	T	82	78	8.00×10^7	8.10×10^7
18	T	T	129	146	1.38×10^8	1.34×10^8
19	T	T	248	233	2.40×10^8	2.29×10^8
20	T	T	362	348	3.55×10^8	4.01×10^8
21	T	T	624	658	6.41×10^8	6.86×10^8
22	T	T	458	422	4.40×10^8	
23	T	T	188	163	1.76×10^8	

* T, too many to count.

Generation time as computed from plate-count populations of the fourteenth and twenty-first hours was 1.30 hours.

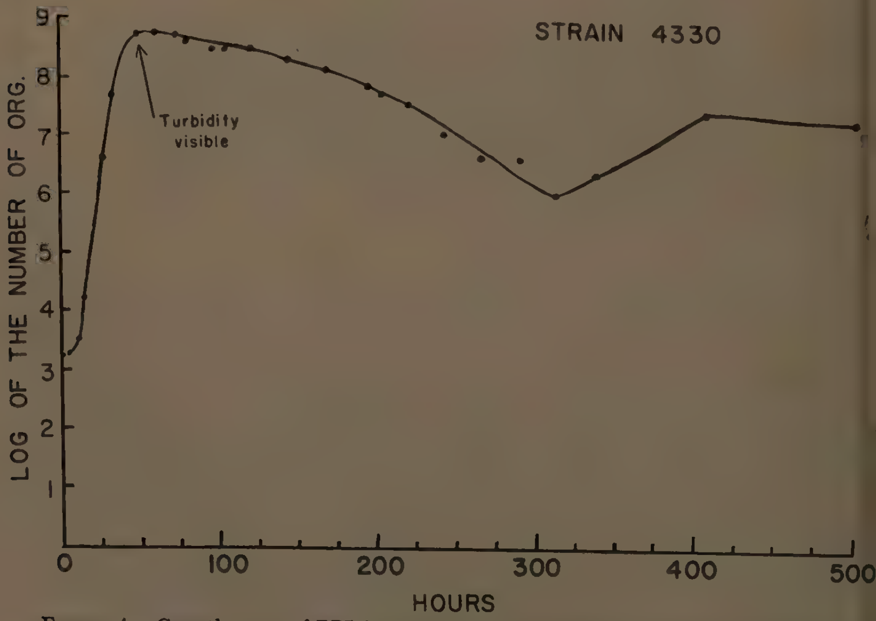

FIGURE 4. Growth curve of PPLO strain 4330 determined by a plate-count method.

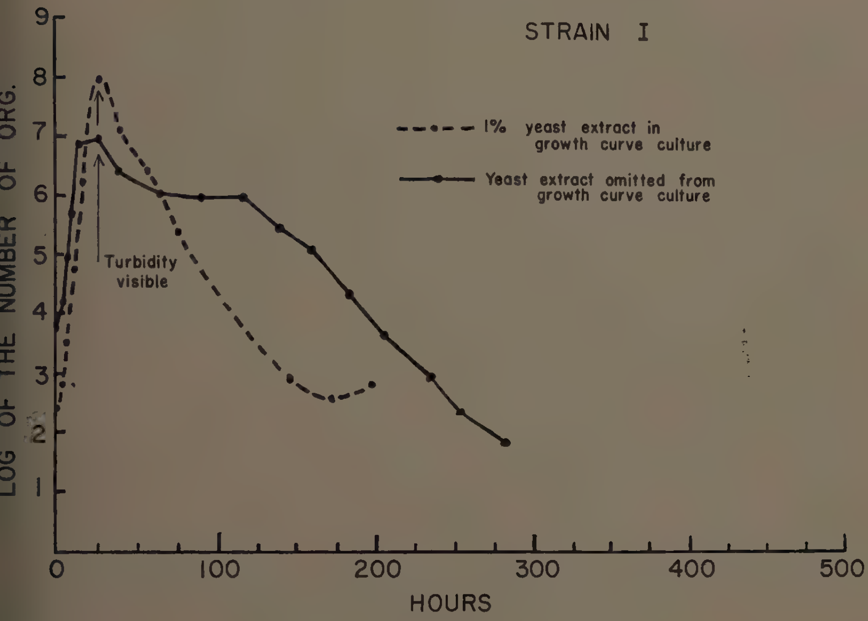

FIGURE 5. Growth curves of PPLO strain I.

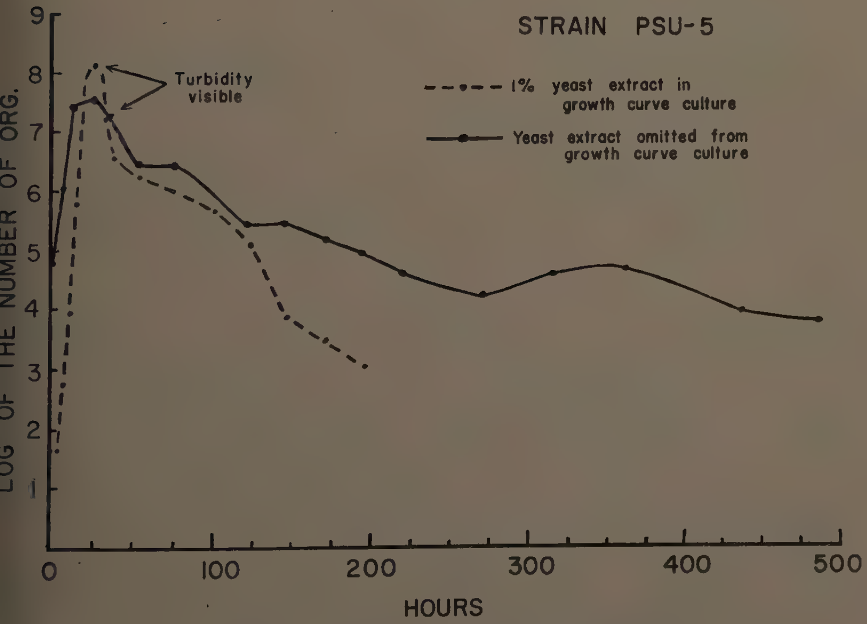

FIGURE 6. Growth curves of PPLO strain PSU-5.

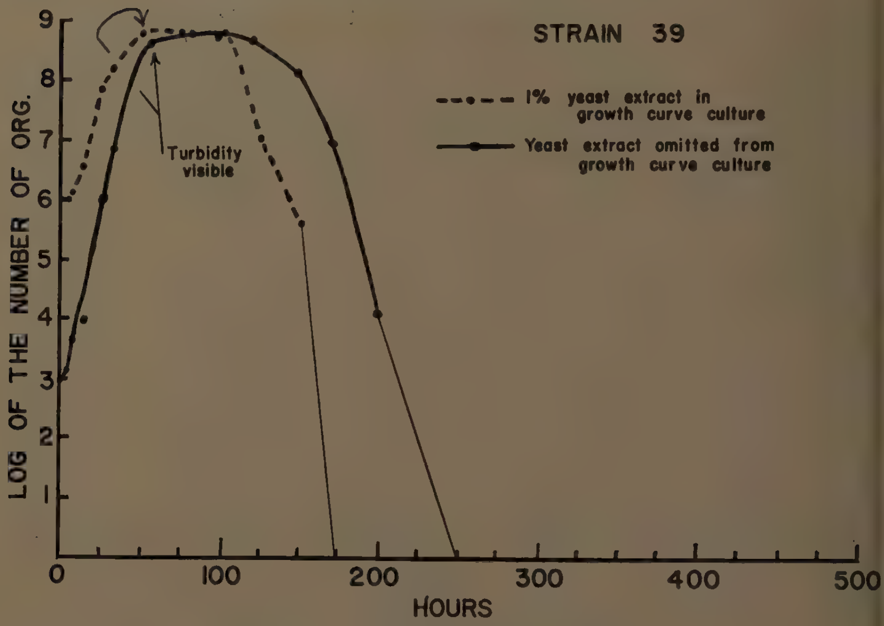

FIGURE 7. Growth curves of PPLO strain 39.

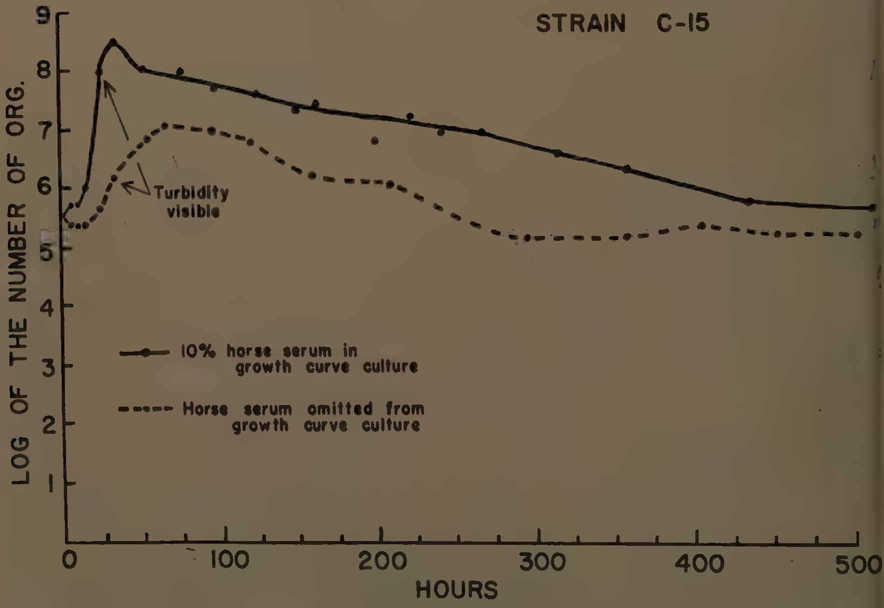

FIGURE 8. Growth curves of PPLO strain C-15 with and without horse serum in the growth-curve cultures.

curves. Comparison of the growth curves with and without horse serum (FIGURE 8) shows that the presence of horse serum, under the conditions of the experiment, enhanced growth.

The data in TABLE 1 show that a relationship was obtained between the populations determined by the plate-count method and the populations computed under the assumption that binary fission was the mode of reproduction during the logarithmic phase of growth of strains 39, I, PSU-5, and 4330.

There was a close relationship between the populations determined by the plate-count assay and the computed populations for the hourly intervals during the logarithmic phase of growth of strain I (TABLE 2). The curve of this determination is shown in FIGURE 3. The growth curve follows a straight line between the fourteenth and twenty-first hours. The generation time of this culture was determined to be 1.3 hours. These results indicate that the population doubled at the generation-time interval, and the mode of reproduction appears to be of the type where one organism gives rise to two daughter cells.

Evidence in favor of binary fission as the mode of reproduction of PPLO has been presented in studies with the electron microscope by Smith *et al.*[5] and again by Tulasne and Bringmann.[6] The results of the experiments reported here show that the growth curves of these PPLO are similar to bacterial growth curves. The analysis of the logarithmic phase of growth of the PPLO presents additional evidence favoring binary fission as the method of reproduction of PPLO.

Summary

Growth curves were determined on five strains of PPLO through a plate-count method that appears to be accurate and requires no special equipment or complex techniques.

The growth curves of all strains showed characteristics of the bacterial growth curve. Analysis of the logarithmic phase of growth shows that the organisms increased in numbers in a geometrical progression and that a common ratio of two could be applied. These data are presented as evidence favoring binary fission as the mode of reproduction of PPLO.

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PPLO CONTAMINATION IN TISSUE CULTURES

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Contamination of tissue-cell cultures by pleuropneumonialike organisms (PPLO) could have profound effects on research in which tissue cells are being studied or used as tools. The study of these tissue-cell contaminants also might yield information as to the nature of PPLO as intracellular parasites and as to their relationship to bacteria.

Robinson *et al.*[1] reported that 3 of 5 lines of HeLa cells and a line of human conjunctival cells routinely grown in the presence of penicillin and streptomycin were contaminated with PPLO. Collier[2] reported that 11 of 14 lines of HeLa cells were contaminated with PPLO.

This paper deals with research directed toward determining the extent and the source of PPLO contamination of tissue cells.*

The method employed to isolate PPLO from tissue-cell cultures was to transfer 1 ml. of the tissue culture containing both the cells and supernatant to 5 ml. of Bacto-PPLO broth[3] enriched with 1 per cent PPLO serum fraction.[4] This culture was incubated aerobically at 37° C. for 3 days, after which 0.2 to 0.4 ml. of the broth culture was inoculated over the entire surface of Bacto-PPLO agar[3] enriched with 1 per cent PPLO-serum fraction. The plate cultures were incubated at 37° C. Colonies of PPLO appeared within 3 to 6 days.

The direct seeding of tissue-cell cultures onto the agar medium did not increase the frequency of positive isolations. Direct plating of tissue cells made it difficult to recognize the colonies of PPLO among the debris of the tissue cells, and the colonies were not as numerous as after one passage in broth.

The inverted Petri-dish cultures are examined by transmitted light under the low power of the microscope at a magnification of ×100. The colonies varied considerably in size and morphology. The diameters of the colonies have ranged from about 0.08 mm. to 0.3 mm. The morphology of the colonies has varied from vacuolated, lacy, to small dense refractile types. After a few passages on agar medium, some of the colonies show the classic "fried-egg" appearance.

To eliminate further the confusion of PPLO colonies with tissue-cell debris or artifacts in the agar, all cultures suspected of being positive for PPLO were subcultured and the strains of PPLO maintained on artificial medium for varying numbers of passages. Frequently the strains of PPLO have not grown well until after 3 or 4 serial transfers on agar medium. Occasionally when a tissue cell line has been cultured repeatedly a negative result has been obtained among the series of positive isolations, which probably reflects unavoidable faults in sampling or cultivation.

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* Samples of tissue-cell cultures were obtained from the South Jersey Medical Research Foundation, Camden, N.J.; Virus Research Laboratories, Children's Hospital of Philadelphia, Philadelphia, Pa.; School of Medicine, University of Miami, Coral Gables, Fla.; Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada; Presbyterian Hospital, Philadelphia, Pa.; and the Medical School, University of Minnesota, Minneapolis, Minn.

Colonies stained with Dienes stain and would not rub off the surface of the
gar. All PPLO contaminants that were isolated required serum or serum
raction for growth.

Forty-one lines of tissue cells were tested for contamination by PPLO, of
vhich 24, or 59 per cent, were found to contain PPLO. Cell lines derived from
uman conjunctiva, kidney, intestine, lung, bone marrow, and liver were posi-
ive. Monkey kidney and mouse mesenchymal cells also were found to con-
ain PPLO. All cells in first and second tissue-culture passage were negative
or PPLO. PPLO were isolated from cells cultivated in the presence of human,
orse, and calf sera. All but three positive cell lines had been grown in the
resence of human serum at some time. Two lines of monkey kidney cells and
line of mouse mesenchymal cells that were never in contact with human serum
ontained PPLO contaminants.

None of the contaminated cell lines demonstrated gross cytopathogenic
ffects, and no turbidity was evident.

Twenty-five samples of sera have been tested, and all were negative for
PLO. These included seven pooled lots of horse serum, two pooled lots of
alf serum, and 16 samples of human serum comprising pooled lots and in-
ividual samples.

The source of the PPLO contaminants in tissue cells is unknown. It has
een suggested (Robinson *et al.*[1]) that the contamination of some common
omponent of the culture media might be the source of the contaminating
PLO. The isolation of PPLO from cells that have been grown only in the
resence of horse serum and from cells grown only in human serum, together
vith the failure to isolate PPLO from human and horse serum, indicates that
erum is probably not the source of the contaminants.

Another suggested source of PPLO in tissue cell cultures is through the con-
amination of cells when they are originally isolated. This hypothesis could
xplain the contamination of some cell lines, particularly those derived from
he human genitourinary tract and oral cavity, but this suggestion would not
xplain the presence of PPLO in cells taken from human bone marrow, liver,
nd intestine and from monkey kidney and mouse mesenchymal cells.

PPLO seem to be most prevalent in those cell lines that have undergone
umerous passages in the hands of many individuals. This would tend to
ndicate that a break in aseptic technique is a likely source of the contamina-
ion. The direct introduction into tissue cell cultures of human PPLO by a
reak in aseptic technique could account for the contamination. Agglutina-
ion tests carried out in our laboratory indicate that the PPLO isolated from
ell cultures are not serologically similar to human type 2 genital strains of
PLO. Further serologic comparisons will help to clarify the role of other hu-
nan strains of PPLO in the contamination of cell cultures.

Another possible source of the contamination is by the formation of stable L
orms of contaminating bacteria. Since penicillin is known to produce L forms
f bacteria and is widely used in tissue culture, and since bacteria are common
ontaminants in cell cultures, it is possible that these PPLO contaminants
epresent stable L forms produced from contaminating bacteria by the action
f penicillin.

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INTRACELLULAR GROWTH OF PLEUROPNEUMONIALIKE ORGANISMS (PPLO) IN TISSUE CULTURE AND *IN OVO**

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INTRODUCTION

The present studies were undertaken about six years ago[1-3] in order to in- estigate some problems of intracellular and extracellular development of leuropneumonialike organisms (PPLO). The PPLO have been incriminated n such diseases of man as nongonococcal urethritis and Reiter's syndrome[4] lthough, at present, it is difficult to establish whether the organisms are re- ated causally to the diseases.[5] PPLO that are apparently pathogenic for hickens and turkeys,[6,7] sheep and goats,[8] swine,[9,10] cattle,[11] rats,[12] mice,[13,14] and logs[15] have been obtained from lesion material from these animals.

Some workers have reported detection of intracellular inclusions that pre- umably were an intracellular phase of the PPLO.[4,16-19] Unfortunately, the ultivation of PPLO from this material to corroborate the presence of the rganisms was not attempted in most instances. In our studies we inoculated nown strains of PPLO into tissue cultures of primary cells and established ell lines and into embryonated chicken eggs. Intracellular growth or survival nd certain interesting extracellular forms have thus been demonstrated.[1-3] fter our initial reports,[1-3] Shepard[20] and Wittler *et al.*[21] reported that certain trains of PPLO could be found intracytoplasmically in PPLO-infected HeLa ells.

Subsequent to our first observations on PPLO intentionally placed into tissue ultures, a number of workers, including one of us (L.H.), have encountered hese organisms as accidental contaminants in tissue cultures.[22-25]

MATERIALS AND METHODS

Organisms

Two of the four poultry strains of PPLO used in this study were isolated rom chickens with chronic respiratory disease (CRD) at New Bolton Farms, chool of Veterinary Medicine, University of Pennsylvania, Kennett Square, a., by James G. Lecce. The strains (J and T) were isolated from the trachea of the birds and carried simultaneously on PPLO agar[26] and in embryonated chicken eggs. The strains fulfill all of the criteria established by Morton *et al.*[27] for inclusion as members of the PPLO group.

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† This work was done, in part, during the tenure of a University of Pennsylvania Scholar- ip, a George Leib Harrison Scholarship, University of Pennsylvania, and a James W. Mc- aughlin Research Fellowship in Infection and Immunity, Department of Microbiology, niversity of Texas Medical Branch, Galveston, Texas.

Chicken strains 1 and 2 and human strains Campo, O7, 39, 48, and 110 also were used. Histories of these strains already have been described.[28]

Strains V-73 and 101 were isolated from human males during a survey of urethritis cases conducted by Don Peoples at the Department of Medical Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pa., in 1955. These strains were isolated first on blood agar and later adapted to grow on PPLO agar.[29] They represent the most recent isolates used in this study.

Preliminary work was done with the PPLO strain J isolated from a chicken before extending the studies to include the human PPLO strains. Strain J was selected in order to establish a homologous tissue culture system consisting of chick heart fibroblasts, chicken plasma, chicken serum, chicken embryo extract, and the PPLO strain J isolated from a chicken.

PPLO used for inocula in all of the tissue culture studies were prepared by cutting out strips of agar containing a heavy growth of PPLO. The strips were cut with a sterile scalpel so as to fit into the barrel of a 5-ml. syringe. The agar was crushed in the syringe and directed into a test tube containing 5 ml. of PPLO nutrient broth.[26] After an hour or so the crushed agar settled, and the supernatant was found to contain numerous viable PPLO elements after plating on agar.

Tissue Culture Materials

Chicken embryo extract (EE_{50}), chicken serum, heparinized chicken plasma, and Hanks' balanced salt solution were prepared, in general, according to the directions of the staff of the Tissue Culture Course.[30] These reagents were tested for the presence of contaminating PPLO before they were used.

Maintenance solution (*MS*). This was prepared by adding one part of ×10 concentrated Hanks' balanced salt solution containing no glucose to 1 part of Parenamine solution.* The Parenamine solution was prepared by mixing 2 parts of the undiluted Parenamine stock with 100 parts of distilled water. The Hanks'-Parenamine combination was diluted further with 8 parts of distilled water. Phenol red was added as a *p*H indicator to a concentration of 0.02 per cent. The MS was autoclaved and stored at 4° C.

Glucose-bicarbonate-penicillin-streptomycin solution (*GBPS*). This solution contained 0.1 gm. of glucose, 0.035 gm. $NaHCO_3$, 6000 U. of penicillin and 6000 μg. of streptomycin per ml.

Glucose-bicarbonate solution (*GB*). This solution contained 0.1 gm. of glucose and 0.035 gm. of $NaHCO_3$ per ml.

Trypsin solution (*Difco trypsin, 1:250*). This was prepared to a final concentration of 0.5 per cent in MS, filtered through ash-free filter paper to clarify it, and then through a Selas filter to sterilize it. The *p*H was adjusted to 7.6 with sterile $NaHCO_3$.

Tissue culture growth media. These media were prepared by combining 5

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* Product of Winthrop Stearns, Inc., New York, N.Y.

arts of MS and 4 parts of serum: chicken serum for the tissue culture experi-
nents using chicken heart explants, and horse serum for the growth of HeLa
nd the chicken heart cells in monolayers. One part of EE_{50} was added, and
he entire medium was centrifuged at low speed to clarify the solution. To
ach 10 ml. of growth medium, 0.2 ml. of one of the following solutions was
dded: GB solution for the tissue culture experiments with PPLO, and GBPS
olution for the chicken heart cell monolayers and HeLa cells carried in bottles.

For studies utilizing the McCoy cell line, the medium consisted of human
scitic fluid, EE_{50}, Gey's balanced salt solution, 100 U. of penicillin, and 100
g. of streptomycin per ml. The antibiotics were omitted when PPLO were
sed in this cell line.

Tissue Culture Methods

Maximow double-slide cultures. Explants, approximately 1 cu. mm., were
repared from the hearts of 9-day-old chicken embryos on coverslips according
o the Maximow double coverslip technique.[31] Chicken plasma was mixed
vith a few drops of PPLO suspension. (For the control explants, the same
mount of PPLO suspension heated for 30 min. at 56° C. was added to chicken
lasma. Subsequent plating on agar of heat-inactivated PPLO suspensions
howed no PPLO colony formation.) Separate explants were drawn up in a
asteur pipette with EE_{50} and placed on individual coverslips containing a
mall drop of the plasma solution prepared as described above. The plasma
nd EE_{50} were mixed quickly with a platinum wire and then drained of almost
ll of the liquid, thus leaving a very thin plasma clot over the explant. The
hin plasma clot permitted cytological observation of the outgrowing cells.
fter the clot had formed, a perforated cellophane disk was placed over the
xplant and one drop each of EE_{50}, serum, and heated or unheated PPLO
uspension was added. The coverslips were sealed in the depression slides with
araffin and incubated at 37° C.

Chicken heart cells in monolayers. Chicken embryo heart cells were estab-
shed for use in the hanging coverslip technique that will be described sub-
equently. Six explants of heart tissue (1 cu. mm.) from a 9-day-old chicken
mbryo were planted on the walls of each of 3 roller tubes. The explants were
mbedded in plasma, and 2 ml. of growth medium were added. In this case
orse serum was used. After 7 days' incubation in a roller drum and periodic
djustments of the *p*H with GBPS, the growth fluid was withdrawn and saved.
he explants with their outgrowths were trypsinized from the test tube walls
nd centrifuged at 1000 rpm for 10 min.; the 0.5 per cent trypsin solution
upernatant was discarded, and the fibroblasts and explants were resuspended
n the 5 ml. of growth medium saved from the roller tubes. The heavier ex-
lants fell to the bottom of the tube and were discarded. The resulting cellular
uspension was run back and forth in a 1-ml. pipette in order to break up
lumps of cells. To this suspension was added an equal volume of fresh growth
nedium, and 5 ml. of the mixture was placed into each of 2 bottles having a
oor area of 25 sq. cm. The bottles were stoppered and incubated at 37° C.
he cells settled in a few hours and, after 1 day, appeared to be in good condi-
ion. After 4 days the medium was replaced with fresh medium. In 10 days

the chicken heart cells covered the floor of the bottle in a monolayer. By using the technique of Scherer *et al.*[32] for the cultivation of HeLa cells, we were able to carry the chicken heart cells for periods as long as 5 months.

Hanging coverslip technique. Coverslips 7 mm. × 43 mm. were cut from larger stock and inserted into 150 mm. × 16 mm. test tubes. Then 0.4 ml. of the chicken heart fibroblast suspension, prepared by trypsinization and resuspension from the bottles as previously described, was put into each tube. The tube was stoppered after the addition of 1 drop of heated or unheated PPLO suspension, and the liquid was allowed to creep between the curved wall of the test tube and the flat surface of the coverslip. The test tube was rotated one-half turn, so that the coverslip hung from the roof of the test tube, held securely by the surface tension of the liquid medium, as shown in FIGURE 1. The cells then fell onto the flat surface of the coverslip and, in a few days grew out into a monolayer. In this technique a monolayer of cells is available, free of a clot, on the surface of a coverslip, where ease of observation and withdrawal for staining and mounting are achieved. Another advantage of this technique is that very small amounts of medium are necessary.

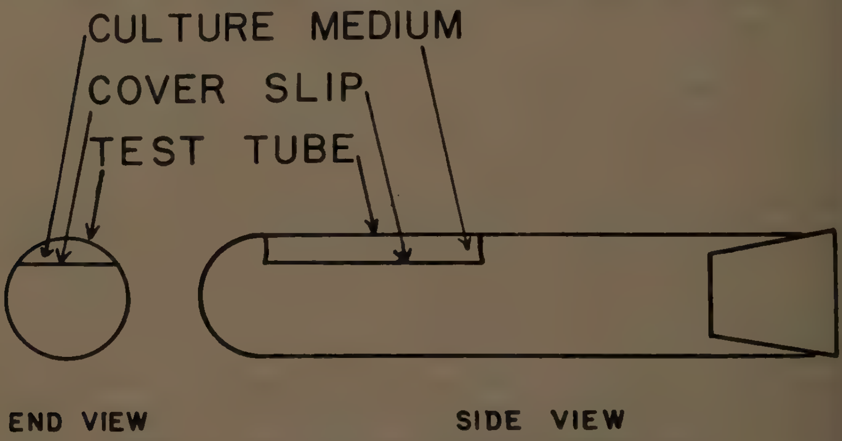

FIGURE 1. System employed in the hanging coverslip method.

HeLa cell cultures. HeLa cells were obtained from Anthony J. Girardi, formerly of the Virus Diagnostic Laboratory, Children's Hospital, Philadelphia, Pa. The stock cultures were carried according to the technique of Scherer *et al.*[32] in a medium consisting of 5 parts of MS, 4 parts of horse serum, and 1 part of EE_{50}. GBPS was added. For experiments with PPLO, the HeLa cells were cultured on hanging coverslips in the above-described medium without penicillin and streptomycin.

Human synovial cells. Since PPLO are reported to have been isolated from joint fluids,[33] an uncloned cell line (McCoy) was used that originally was isolated from the synovial fluid of an adult patient suffering from a joint pain of unknown etiology. This cell line was established in the laboratory of C. M. Pomerat, University of Texas Medical Branch, Galveston, Texas. The cells are fibroblastlike. They were cultured on flying coverslips in Porter flasks in a medium consisting of human ascitic fluid, Gey's balanced salt solution, and EE_{50}. All reagents were tested for contaminating PPLO, and no antibiotics were used in the experiments. Only the human PPLO strains were used with the McCoy cell line. Control cultures received heat-inactivated PPLO. Coverslips were removed periodically, after additions of the PPLO, and stained.

Staining procedures. Either one of two staining procedures was used throughout these studies: the May-Grünwald and Giemsa stain as described by Jacobson and Webb[34] or a modification of the Macchiavello staining method. We observed that a careful acetone extraction of Macchiavello-stained preparations and subsequent clearing in xylol revealed some of the elements of the PPLO as purple-colored material, whereas the tissue culture cells stained blue.

Embryonated Egg Methods

Determination of best age and route of inoculation. Poultry PPLO strains 1 and 2 were used to determine the best age and route of inoculation of embryonated chicken eggs. Keller and Morton[35] determined that the chorioallantoic and amniotic routes of inoculation with human PPLO strains gave inconclusive results in respect to embryo mortality or injury to the newly hatched chick. We inoculated PPLO via the yolk sac route, and compared this route with the chorioallantoic route of inoculation. Embryonated chicken eggs that were 4, 6, 7, 8, 9, 11, and 12 days old were inoculated by both routes with 0.2-ml. broth suspensions of PPLO strains 1 and 2. The criterion used for the best route and age for inoculation was the greatest number of embryo deaths observed in the shortest period of time postinoculation.

Serial passage in yolk sacs. Undiluted yolk (0.2 ml.) was passed into the yolk sacs of 7-day-old embryonated eggs. Human strains Campo and O7 were passed 8 times over a period of 7 months. Chicken strains J, T, 1, and 2 were passed 14 times over a period of 1 year. Yolk from each passage was stored at −20° C. This study was undertaken in an attempt to increase the virulence of the PPLO. The infected yolk was saved in order to determine the stability of PPLO in yolk at −20° C.

Another purpose of this work with embryonated eggs was to confirm *in ovo* some of the cytological observations made in tissue culture. Smears from yolk sac stalks were made to determine the presence of inclusions in cells from PPLO-infected eggs.

RESULTS

Maximow Cultures with PPLO Strain J

Observations of chicken-heart explants cultured by the Maximow technique n the presence of PPLO, strain J, revealed, by modified Macchiavello staining, numerous areas of purple-staining material. These explants, embedded in plasma clots, retained the extracellular PPLO in the clots. The observation that PPLO will grow in embryo extract already has been reported.[36] In the plasma clots, PPLO grew much as they do on agar, that is, as discrete colonies with darker centers (FIGURE 2*d*). The darker colony centers, intracellular PPLO, and extracellular saclike structures stained a deep purple with the modified Macchiavello stain. No cytopathogenicity was observed in tissue

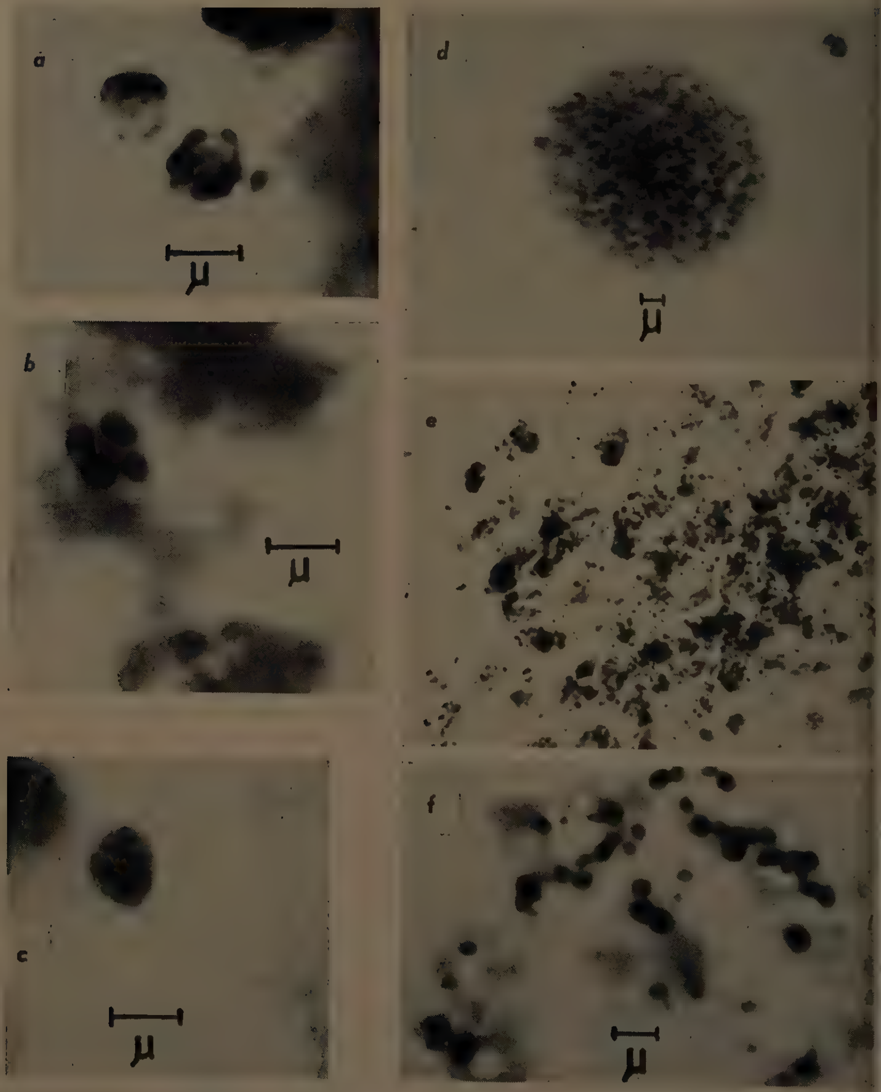

FIGURE 2. (*a* and *c*) Extracellular saclike structures found in plasma clots of chick embryo heart explants inoculated with PPLO, strain J. Modified Macchiavello. (*b*) Cluster of four extracellular PPLO units found in plasma clots of chick embryo heart explants inoculated with PPLO, strain J. Modified Macchiavello. (*d*) A small PPLO colony in a plasma clot found in a Maximow double-coverslip preparation. Central granules in the colony are purple; periphery is blue. Modified Macchiavello. (*e*) Low magnification of a large area of yolk sac cells from an embryo dead of infection with PPLO, strain J. Small intracytoplasmic inclusions appear to lie about many of the cell nuclei. May-Grünwald and Giemsa. (*f*) A single yolk sac cell parasitized with PPLO, strain J. May-Grünwald and Giemsa.

cultures of heart cells carried up to seven days and, in no case, was purple-staining material observed in control cultures.

The sequence of events was observed by staining a series of Maximow cultures at intervals over a 4-day period. A number of morphologically different structures that appeared to develop in several stages were revealed. The size of the units was estimated to range from 0.3 to 0.8 μ. At first, small (0.3 μ) intracytoplasmic spherical particles were observed; later, when such particles were found to be enlarged, sacs containing from 4 to 10 dark purple-staining areas could be seen extracellularly. Clusters of these sacs were found in the thin plasma clot, but were not observed intracellularly (FIGURE 2*a* to *c*).

Hanging Coverslip Cultures with PPLO Strains J and Campo

The hanging coverslips were prepared in order to be certain that the intracellular particles observed in the Maximow preparations were indeed intracellular and not embedded in the plasma clot overlying a particular cell. No extracellular elements were found in the hanging coverslip preparations, presumably because they were lost when the chicken cells were bathed in a fluid medium during growth and washed before staining. Intracytoplasmic inclusions were found (FIGURE 3*a* and *b*). These stained red to purple with the May-Grünwald and Giemsa stain. The inclusions were discrete units approximately 0.5 μ in size, and sometimes they tended to lie adjacent to the nuclei of the fibroblasts. Parasitized cells usually were found in groups with many unparasitized fibroblasts on each coverslip. The best preparations contained no more than 5 per cent parasitized chicken cells. Control cultures containing heat-inactivated PPLO had no red- or purple-staining intracytoplasmic material (FIGURE 3*c*).

Cytological Studies of Embryonated Eggs Dead of Infection with PPLO Strains J, 1, and Campo

Similar results also were observed in the stained yolk sac cells of embryonated eggs dead of infection with PPLO strains J, 1, and Campo. May-Grünwald and Giemsa stains of this material revealed numerous areas of heavily parasitized cells (FIGURES 2*e* and *f* and 3*d*). Red-staining elements also were found in agar colonies of PPLO stained by this method. Yolk sac cells from uninfected controls showed no such results. The size range of the yolk sac cell inclusions was from 0.3 to 0.8 μ.

HeLa Cell Hanging Coverslip Cultures

Strains J and Campo did not produce intracytoplasmic inclusions in HeLa cells. The PPLO grew freely in the tissue culture growth media.

Porter Flask Cultures of Human Synovial Cells (McCoy) in the Presence of PPLO

All human strains of PPLO tested in the presence of this cell line produced numerous intracytoplasmic inclusions after 3 to 4 days' incubation (FIGURE 4*b*, *c*, and *d*). Controls were negative. The inclusions from these strains (Campo, 48, 39, O7, 110, 101, and V-73) stained a deep red with the May-Grünwald and Giemsa stain. After 3 days a slowly developing cytopathogenic effect was observed that terminated in the total destruction of the cells in about 5 days. The cytopathogenicity was marked by the rounding of the cells, increasing cytoplasmic granularity, and ultimate nuclear pyknosis. Complete cell degeneration and lysis eventually resulted. Strains V-73 and 101 formed intracytoplasmic inclusions in 30 per cent and 40 per cent, respectively, of the cells after 3 days.

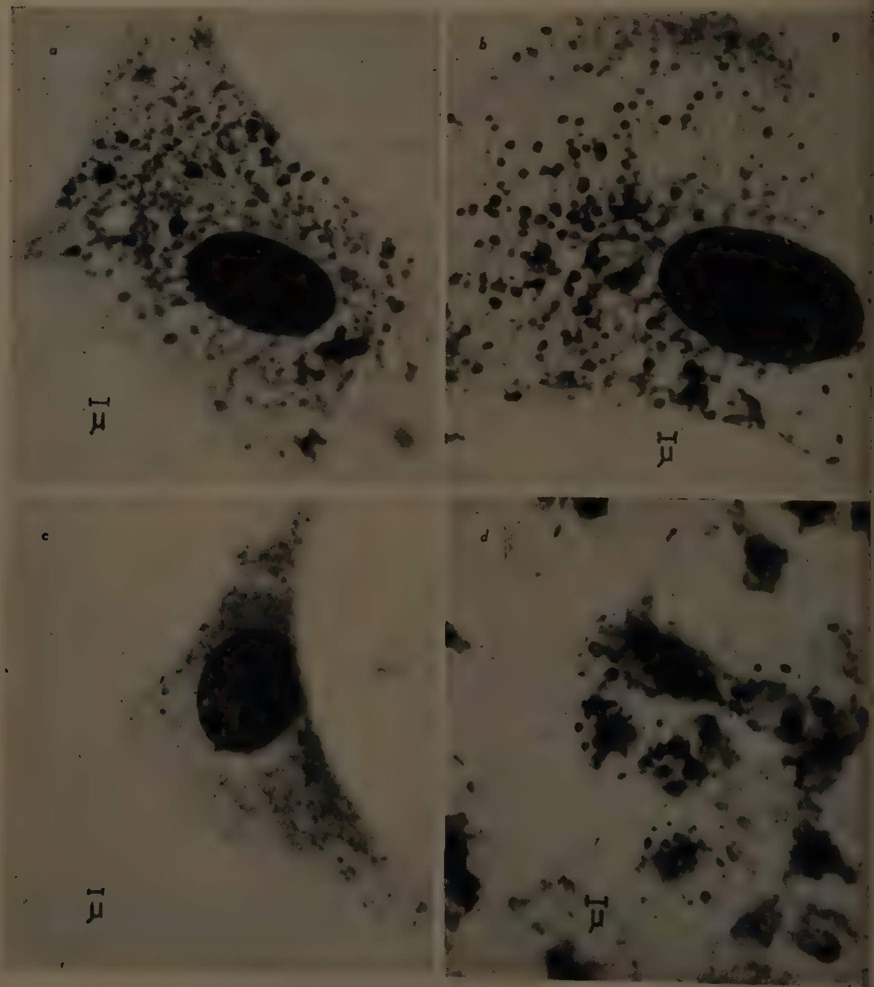

FIGURE 3. (*a* and *b*) A single chicken heart fibroblast grown by the hanging coverslip technique. The cytoplasm of each cell contains numerous strain J PPLO inclusions. May-Grünwald and Giemsa. (*c*) A single chicken heart fibroblast grown by the hanging coverslip technique. This is from a control culture containing heat-inactivated PPLO, strain J. May-Grünwald and Giemsa. (*d*) A cluster of yolk sac cells, five of which show distribution of PPLO inclusions about the nuclei. May-Grünwald and Giemsa.

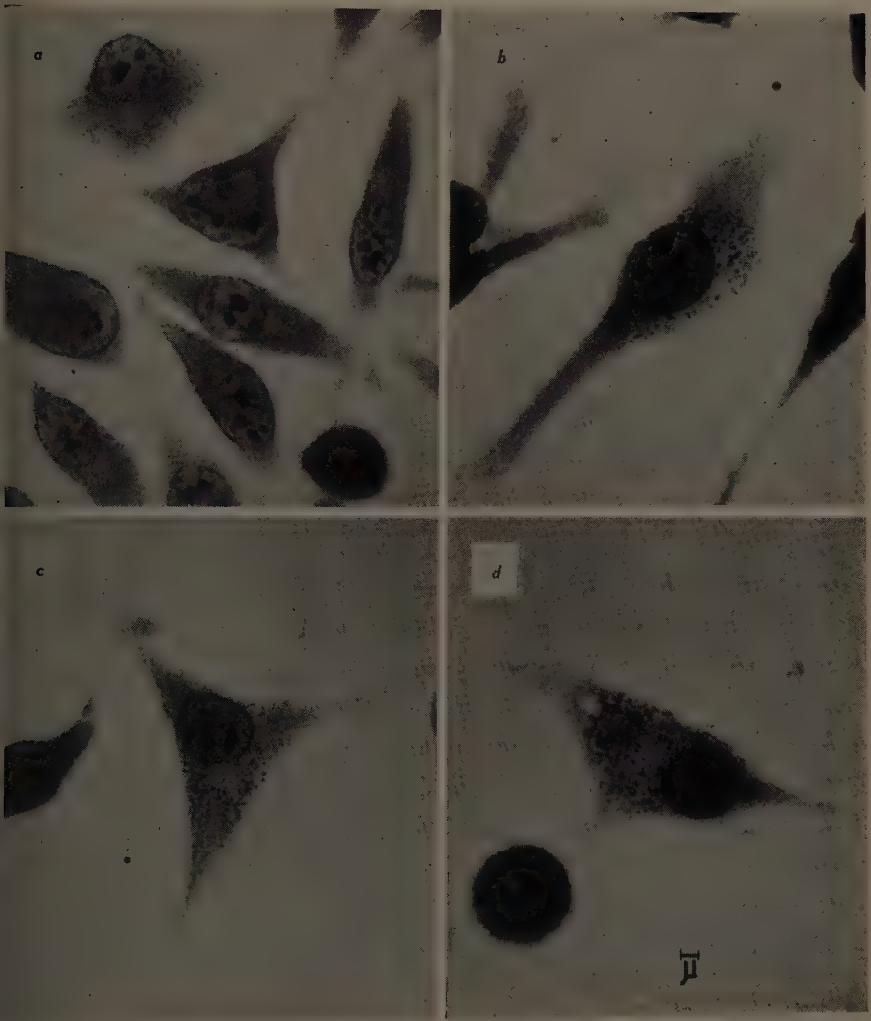

Figure 4. (*a*) Tissue culture, cell line McCoy isolated from the synovial fluid of a patient suffering from a joint pain of unknown etiology. This preparation contains heat-inactivated PPLO and serves as a control. May-Grünwald and Giemsa. (*b*, *c*, and *d*) McCoy cells showing intracytoplasmic inclusions after inoculation with PPLO strains Campo, 48, and 39, respectively.

Determination of Best Age and Route of Inoculation of Embryonated Eggs

Eggs inoculated with poultry strain 1 or poultry strain 2 via the chorioalantoic route either hatched normally or, as in most cases, died a few days beore they were scheduled to hatch. There was no obvious gross pathological hange.

Inoculation of eggs via the yolk sac route with either strain killed the embryo in from 2 to 4 days if the embryo was 7 days old at the time of inoculation.

Yolk sac inoculations at 8, 9, 11, and 12 days of age showed embryo mortality in from 5 to 9 days postinoculation. Embryonated eggs inoculated via the yolk sac that were younger than 7 days gave inconclusive results. Therefore, according to the criterion of the greatest number of embryo deaths in the shortest period of time, a 7-day-old embryo inoculated via the yolk sac route was the most susceptible.

On one occasion when the best route of inoculation was being determined, eggs inoculated via the chorioallantoic route with strains 1 and 2 hatched live chickens. The chicken receiving strain 1 was killed after 8 days, when it had developed a noticeable "rattle" and wing lesion. Smears from various organs on agar showed no PPLO. A mucous discharge from the trachea contained numerous PPLO, as indicated by plating on agar. The second chicken inoculated with PPLO strain 2 took 5 days to break out of the shell and was crippled. There were no apparent lesions or edema of the legs. A very noticeable rattle and sucking noise could be heard as the animal breathed. On dissection much mucous was found in the trachea, and PPLO were isolated.

It is interesting to note that inoculations of PPLO into the yolk sacs of 7-day-old embryonated eggs caused the typically erratic death pattern and extensive cutaneous hemorrhages of the embryos previously observed by other workers[17,37] in embryos with CRD. Similar results were obtained by Delaplane[38] and by Hoyt *et al.*,[39] although at the time these workers were unaware that the agent under investigation was a PPLO.

Serial Passage of PPLO Strains in Embryonated Eggs and Yolk Stability of PPLO at −20° C.

The mortality rate of embryos upon passage of each strain (J, T, 1, 2, Campo, and O7) in the yolk sacs of embryonated chicken eggs was very erratic. From 50 to 100 per cent of embryos died regardless of the passage. If death occurred, it happened 2 to 6 days postinoculation (4 to 10 days postinoculation with strain T). The interval before death did not decrease with increase in the number of passages. Embryos dead of infection with PPLO of any human or poultry strain showed typical gross pathological changes. The skin of the embryo was completely hemorrhagic, and a generalized edema was apparent (FIGURE 5*b*). The hemorrhagia extended over the entire cutaneous area of the embryo with the exception of the tips of the extremities and the top of the head.

The fourteenth passage of yolk material of strain J failed to give an infectivity rate higher than 5 per cent in tissue cultures of chicken heart fibroblasts. The other strains were not tested.

Stability studies on all strains passed in yolk sacs and refrigerated in yolk at −20° C. showed that in strains J, T, 1, and 2, refrigerated for more than 2 years, the number of viable colony-forming units dropped in 2 years from an arbitrary 4^+ to 2^+. A similar drop occurred in strains Campo and O7 after 18 months' storage in yolk at −20° C.

Interpretation of Staining Reactions

The basophilic PPLO units are capable of retaining the basic fuchsin after citric acid decolorization in the Macchiavello staining technique. Counterstaining with methylene blue, bound also by these units, therefore makes the PPLO units purple against the blue background of the remaining tissue culture cytoplasm. The acetone extraction, which is controlled by periodic microscopic examination, extracts much of the methylene blue, yet the strongly basophilic PPLO units retain the deep blue color.

The PPLO colonies, which grow freely in the plasma clot and re-

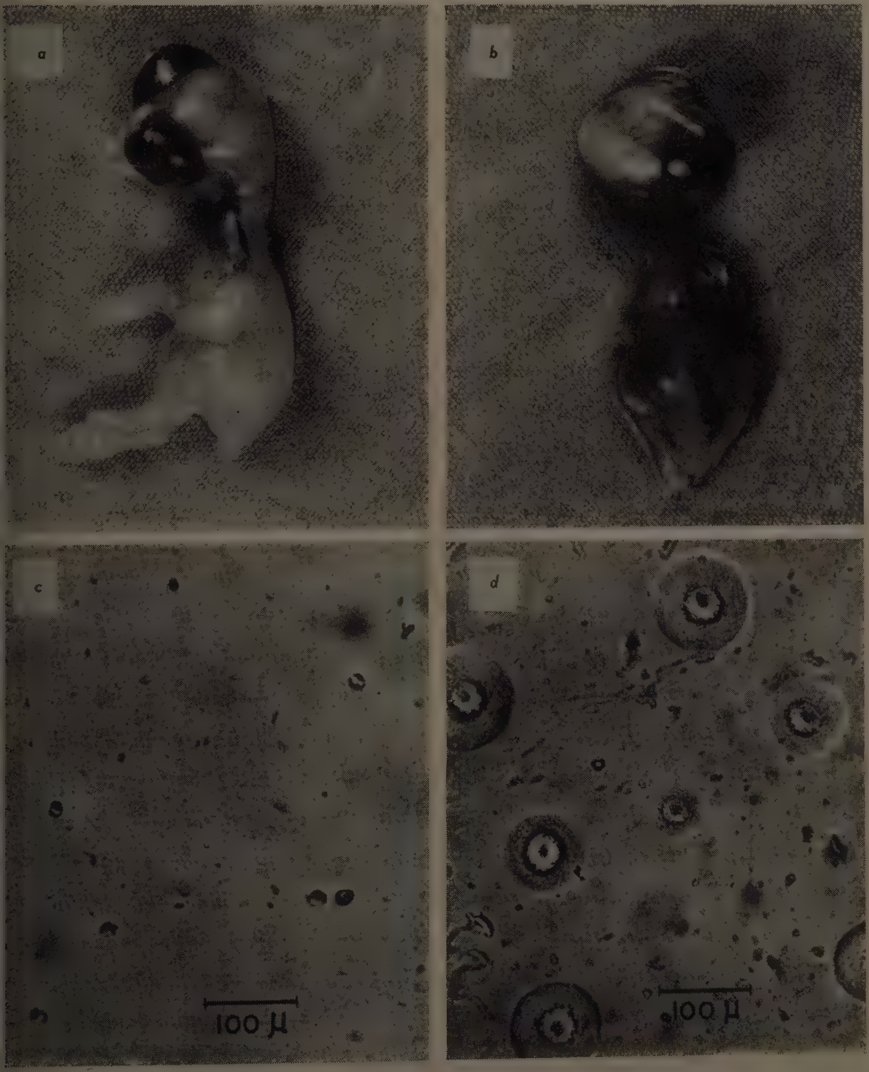

FIGURE 5. (*a*) Eleven-day-old chick embryo uninoculated with PPLO. (*b*) Eleven-day-old chick embryo dead of infection with PPLO strain 1, showing cutaneous hemorrhages. (*c*) Very small PPLO colonies of strain WI-1 isolated from a tissue culture of Human Amnion Fernandes. (*d*) A later subcultivation of PPLO strain WI-1 isolated from tissue culture and showing the typical PPLO colony morphology.

semble colonies seen on agar, exhibit at least two distinctly different kinds of units in respect to staining qualities. The purple units are concentrated in the center of the colony, and blue-staining units are found in the periphery (FIGURE 2*d*). Young colonies contain only the purple units. There is thus some evidence that the purple units give rise to the complete colony. The blue-staining periphery may contain nonviable elements, for only purple-staining units were found intracytoplasmically when Macchiavello's stain was used. It may well be that these are the replicating PPLO units. It must be mentioned, however, that blue-staining PPLO units that may be intracellular under the conditions of the experiments and the staining may not be discernible against the pale blue background of cytoplasm.

Hanging coverslip and Porter flask preparations usually were stained with the May-Grünwald and Giemsa stain, although intracellular units were observed in the hanging coverslip preparations with the acetone-extracted Macchiavello stain. The May-Grünwald and Giemsa stain increased the contrast of the PPLO units against the cytoplasmic background. PPLO units appeared to be red against a blue background. No purple-staining material (modified Macchiavello stain) or red-staining material (May-Grünwald and Giemsa stain) was found in the control cultures of chick heart fibroblasts, Hela cells, or human synovial cells.

DISCUSSION

These experiments on PPLO in tissue cultures have demonstrated that the strains used are capable of an intracytoplasmic existence in certain cells. One of the strains, PPLO strain J, also exhibits a developmental cycle during intracytoplasmic residence. The first units observed are small (0.3 μ) spherical bodies similar in appearance to some virus elementary bodies or to the minimal reproductive units described by Cuckow and Klieneberger-Nobel.[40] These PPLO units seem to enlarge for about 3 days after inoculation. One or two days later, larger (0.9 μ) extracellular saclike bodies appear, containing areas that stain deeply purple with the modified Macchiavello stain.

That the intracellular forms seen in tissue culture preparations are PPLO seems likely because these forms are similar in tinctorial qualities to known PPLO growing extracellularly in the plasma-embedding medium (in the case of strain J) or growing on the surface of PPLO agar. Furthermore, chicken embryos dead of an infection with PPLO strain J exhibited similar forms in preparations from the yolk sac.

In our observation of yolk sac cells we have found corroborative evidence for the intracellular nature of PPLO strain J, but we have not been able to find all the stages that can be seen in tissue cultures. Thus far we have been able to observe only the stage comparable to elementary bodies. Chute and Cole[17] observed 9 extracellular dark-staining round bodies within a thin membrane in a stained chorioallantoic membrane smear. These may represent one of the yolk cell extracellular stages that we have not yet found.

The relative susceptibility of tissue culture cell lines to PPLO used in this study demonstrates the possible existence of a tissue culture spectrum in respect to parasitization with PPLO, not unlike tissue culture spectra for certain viruses.

HeLa cells showed no inclusions with strains J and Campo. In corroboration of our findings, it recently has been reported[22,23] that PPLO are capable of infecting HeLa cell tissue cultures in such a manner as to remain almost unobservable in unstained tissue culture cells. Culturing of the nutrient fluid and, according to our evidence, of the cells themselves is necessary for positive identification. Wittler *et al.*,[21] in confirmation of our work, also found no degeneration of HeLa cells infected with PPLO without the addition of a yeast extract. Wittler *et al.* and also Shepard[20] were able to see intracytoplasmic granules in Giemsa-stained preparations of HeLa cells infected with PPLO. We failed to find such granules in the HeLa cells with which we worked three years prior to the report of Wittler *et al.*[21] and five years prior to the report of Shepard.[20] These differences could be explained in one or more of the following ways: (1) HeLa cells may, in the course of years, have changed in their capacity to support the intracellular residence of PPLO; (2) various sublines of HeLa may be variable in respect to this capacity (sublines of other cell lines similarly may be variable); and (3) strains of PPLO may vary in capacity to infect HeLa cells.

Previous reports[4] of the presence of PPLO in urethral smears may have some validity in light of these tissue culture experiments. Subsequent to our reports[1-3] on the intracellular growth of PPLO in tissue culture, Shepard[41] was able to show, with concomitant isolation of PPLO, that these organisms could be found intracytoplasmically within epithelial cells from male patients with nongonococcal urethritis.

Recently one of us (L.H.) has had experience with a PPLO contaminant isolated from a tissue culture cell line. Since our first report[1] four years ago on the ability of some PPLO strains to grow in tissue culture cells we have constantly surveyed cell lines that have come into our hands for the presence of these organisms. Our technique has been to inoculate a few drops of tissue culture supernatant fluid containing some cells into PPLO broth.[26] After 5 or 6 days' incubation, the broth is streaked on agar, incubated another 5 or 6 days, and examined microscopically for PPLO growth.

A line of tissue culture cells (uncloned) derived from human amnion[42] was found to have PPLO in the supernatant fluids. This cell line (Amnion Fernandes) grew as rapidly as uncontaminated sublines, although there was a suggestion of increased cytoplasmic granularity. The contaminating PPLO, called strain WI-1 (Wistar Institute), were easily isolated on agar after broth passage, but could not be passaged by the usual method of rubbing an agar block containing PPLO colonies over a fresh agar plate. The broth was inoculated into the yolk sacs of 7-day-old embryonated eggs; the embryos, however, hatched normally (Yamamoto *et al.*[43] also have observed the profuse multiplication of PPLO *in ovo* without concomitant embryo mortality). Yolk-sac inoculations were repeated, and the embryos were killed when 12 days old. The yolk and amniotic-allantoic fluids were harvested and stored at −20° C. Before freezing, the material was streaked on agar. All of the amniotic fluids were positive for PPLO, and only an occasional yolk was positive. Control eggs inoculated with sterile broth were negative for PPLO. After 2 weeks the positive fluids were thawed and streaked on agar. The results, unlike those with strains J, T, 1, Campo, and O7, were negative. WI-1, freshly isolated from tissue culture as a contaminant, did not have the stability at −20° C. of PPLO that had been carried for years on agar.

The colonies of WI-1 did not exhibit the typical "fried-egg" appearance after isolation from tissue culture. They were extremely small and were embedded totally in the agar (FIGURE 5*c*). They were typical of those described by Shepard[44] as T-form colonies. They were about 15 μ in diameter with irregular borders and were devoid of a growth zone on the agar surface. The entire colony was embedded in the agar. One of the agar plates with these very small colonies (a plate prepared from one of the amniotic-allantoic fluid preparations before freezing) contained, after about 7 days' incubation, a number of larger colonies that had the classic PPLO morphology, that is, they had the fried-egg appearance and were much larger than the colonies observed before (FIGURE 5*d*). We attempted to pass these colonies onto fresh agar plates without an intervening broth passage, and found that these larger colonies transferred very easily. The same plate also had numerous small colonies. The small colonies obtained on other agar plates from other allantoic-amniotic fluid preparations still failed to pass from agar plate to agar plate. It has been our experience in the past with other PPLO strains that passage from agar to agar of small colonies resembling those just described was as easy as the passage of larger colonies with the fried-egg appearance, although Shepard[44] encountered considerable difficulty in propagating the T forms. The supposition by Shepard that the T forms represent the most dependent state of parasitism and the fact that these T forms were isolated by him in association with cellular elements fits in well with our isolation of these tiny colonies from tissue culture cells.

The importance of the intracellular residence and growth of PPLO cannot be overemphasized. Chang[24] has indicated recently that some sublines of HeLa and a strain of conjuctival cells used for metabolic studies were contaminated with PPLO. Antibiotics that are effective against extracellular organisms may be impotent against the intracellular growth of the same organisms.[45-47] This may account for the isolation of contaminating PPLO from some mammalian cell lines maintained continuously *in vitro*.[22-25] It is possible that certain concentrations of antibiotics of the tetracycline group in tissue culture media may eliminate these organisms from the media and thus drive them into intracellular residence. The experience of Collier[23] might well be explained on this basis.

Since PPLO are susceptible to antibiotics of the tetracycline group,[48,49] the suggestion by Robinson *et al.*[22] that antibiotics of this group be incorporated routinely into media used for the cultivation of tissue cultures may have some merit in respect to elimination of PPLO from the growth media only. Very little attention has been given, however, to the possibility that L forms in tissue cultures may arise if high concentrations of penicillin are present or if there are other environmental conditions that are as yet poorly understood.[50] Virologists routinely use concentrations of penicillin up to 1000 U./ml. when attempting to isolate virus from such material as feces. Some tissue culturists, as a matter of routine, carry cell lines in penicillin concentrations approaching this level. It is known that a number of bacterial genera are capable of reverting to L forms at penicillin concentrations even lower than 1000 U./ml.[51] Since almost all bacterial genera are capable of forming L forms,[50] this possibility must be considered in explaining how tissue culture cell lines have become contaminated with PPLO. Coriell *et al.*[52] have recognized the problem of PPLO contamination in their studies of a serologically reactive component common to several cell lines *in vitro*. A much greater awareness of this variable in cytological and metabolic studies seems to be warranted. We feel strongly that until each investigator can eliminate completely the possibility that contaminating PPLO has influenced his experimental results, all interpretation of such results is suspect.

SUMMARY

Intracellular and extracellular growth of PPLO was observed in tissue cultures of various primary cells and cell lines, and in embryonated chicken eggs. Suggestions of developmental stages were noted with one chicken strain of PPLO grown in chick heart fibroblasts. The stability of PPLO in yolk material that was frozen at −20° C. also was studied. Indications were that PPLO survive in this environment for at least 2 years. Human PPLO were found to parasitize a human line of tissue culture cells derived from synovial tissue. A cytopathogenic effect was observed that led ultimately to complete cellular degeneration. HeLa cells showed no inclusions upon exposure to the PPLO studied, although these organisms grew in the growth medium. Studies are described also of a contaminating PPLO that was isolated from a tissue culture cell line derived from human amnion. The significance of the capacity for some PPLO to reside intracellularly is discussed.

ACKNOWLEDGMENTS

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THE BEHAVIOR OF MURINE PPLO IN HeLa CELL CULTURES

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The report by Robinson *et al.*[1] in 1956 that HeLa cell cultures were contaminated by pleuropneumonialike organisms (PPLO) has been verified in numerous laboratories. Growth of these organisms in a cellular medium was of particular interest to me by reason of my earlier observations on the PPLO associated with chronic coryza of the domestic fowl.[2] In 1936 the avian organisms, then descriptively termed coccobacilliform bodies, were first cultivated *in vitro* in a medium composed of minced 10-day chick-embryo tissue suspended in Tyrode's solution.[3] The organisms were demonstrable in Gram-stained films of both the fluid and the sedimented tissue. They were readily maintained by serial transfer. Viable tissue cells were not essential for growth of the organisms, since multiplication also occurred in the presence of heat-inactivated chick tissue. This medium, which likewise supported the growth of murine PPLO, was later replaced by the more easily prepared serum bouillon.

In recent years, most of the work with PPLO in our laboratory has been limited to cultures isolated from mice and rats. In 1957 experiments with the murine organisms were begun with a view to comparing their growth in the HeLa cell cultures with that in the conventional serum bouillon. Observations were made with cultures of the catarrhal type recently isolated from both naturally and experimentally infected mice. Infectious catarrh has been maintained for many years in specific pathogen-free mice of the Princeton strain by nasal instillation or by contact. Cultures of the murine organisms were routinely carried in heart infusion agar or bouillon enriched with 20 per cent horse serum. The *p*H of the uninoculated medium was about 7.8.

A wild HeLa cell line* was used in this work. The seed cultures were grown in rubber-stoppered bottles containing 8 ml. of nutrient fluid, composed of 20 per cent filtered human serum, 65 per cent Hanks' solution, and 15 per cent tryptose phosphate bouillon (Difco). In the screw-capped tube cultures 1 ml. of fluid was used, and the human serum was replaced by horse serum after the first renewal. Phenol red, 100 units of penicillin, and 100 μg of streptomycin per ml. were added to both solutions.

The Growth of Murine PPLO in Slanted Tubes of Serum Bouillon and Tissue Culture Fluid

Cultures of the murine PPLO in serum bouillon are maintained in cotton-plugged tubes containing 5 ml. of the medium and incubated at 37° C. for 48 hours in an upright position. Since this environment was quite different from that imposed by the tissue culture technique, preliminary observations were made on the growth of the organisms in slanted screw-capped tubes containing either 1 ml. of horse serum bouillon or tissue culture fluid. In these tests and those that follow, the inoculum was 0.01 ml. of a 24- to 48-hour-old PPLO culture in serum bouillon. In some instances penicillin was added to the inoculated tubes, but streptomycin was omitted. Transfers were made with a 3-mm. loop to horse serum-agar plates at intervals of 24, 48, and 72 hours. All plates were sealed with Scotch Tape and examined at a magnification of ×100 after incubation for 5 to 7 days at 37° C.

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* Originally obtained through the courtesy of Sonja Buckley of the Rockefeller Foundation Virus Laboratory, New York, N.Y. It has been ably maintained in our laboratory by Susan Richards.

The results of these tests indicated that the survival rate of the murine PPLO was considerably lower in the tissue culture fluid than in serum bouillon. The plate count, estimated roughly by the number of colonies per microscopic field, showed no significant difference between upright cultures containing 5 ml. of serum bouillon and slanted cultures with 1 ml. The number of colonies averaged several hundred per field through the third day. In the tubes containing 1 ml. of tissue culture fluid, the growth at 24 hours was equivalent to that in serum bouillon. At 48 hours, however, there was a marked decline to 15 colonies per field and, at 72 hours, a further decline to 3 colonies.

PPLO of the catarrhal type produce acid during their growth in serum bouillon. By the third day the *pH* drops from 7.8 to about 7.2. In the tissue culture fluid, which is not as well buffered, the *p*H declines to 6.2. The decreased number of viable organisms in the latter medium may be due in part to the increased terminal acidity.

The Growth of Murine PPLO in HeLa Cell Cultures

The preceding observations with the murine PPLO were then extended to their growth in HeLa cell cultures. Tubes with well-established monolayers were employed. Fluids that contained streptomycin were removed; the cell column was washed once with Hanks' solution; and fresh fluid, with or without penicillin, was added. After inoculation the cultures were examined daily on the stage of the microscope at a magnification of ×100. Cultures from the fluid were made at intervals of 2 to 3 days on serum-agar plates. Fluids that showed tissue cells on examination with the phase microscope were removed and spun at low speed, and Wayson-stained films were made from the sediment.

The murine PPLO multiplied in the HeLa cell cultures and were maintained serially for a number of generations by the transfer of fluid from tube to tube. Extracellular organisms were demonstrable microscopically in the fluids and intracellular groupings within HeLa cells. The number of colonies on the inoculated plates varied considerably, but rarely exceeded 50 per microscopic field. In general, growth was somewhat better sustained than that in the tissue culture fluid alone, but was inferior to that in serum bouillon. In some instances erratic results characterized by the failure of either the cells or the organisms to grow were obtained.

The catarrhal type of PPLO used in these tests presumably corresponds with the species classified as *Mycoplasma pulmonis* in the Bergey *Manual* of 1957.[4] The introductory description lists the organisms of this genus as nonmotile. This statement is incorrect insofar as murine PPLO of the catarrhal type are concerned. Andrewes and Welch,[5] in 1946, were seemingly the first workers to observe motility in fluid cultures of this type. They noted that globules with an attached stalk showed a steady forward movement and that others with a short projection rotated. Motility was reduced on continued cultivation, but was detected up to the sixth transfer. Dark field microscopy was used in these examinations.

Most but not all of the catarrhal strains isolated in our laboratory have shown motile elements on examination with the phase microscope. PPLO of this type are highly pleomorphic. In fluid cultures they show a variety of rods with or without attached spheres, single spheres of varying size, and small mixed aggregates. The term glider has been used to designate the motile rods which may move rapidly across a given microscopic field. The rotating spheres with a single short stem are termed spinners. The number of motile elements varies considerably with different strains, but is usually small in relation to the total numbers of elements. Motile forms and also all rod-shaped components decrease in number on serial transfer and may virtually disappear by the tenth subculture or even earlier.

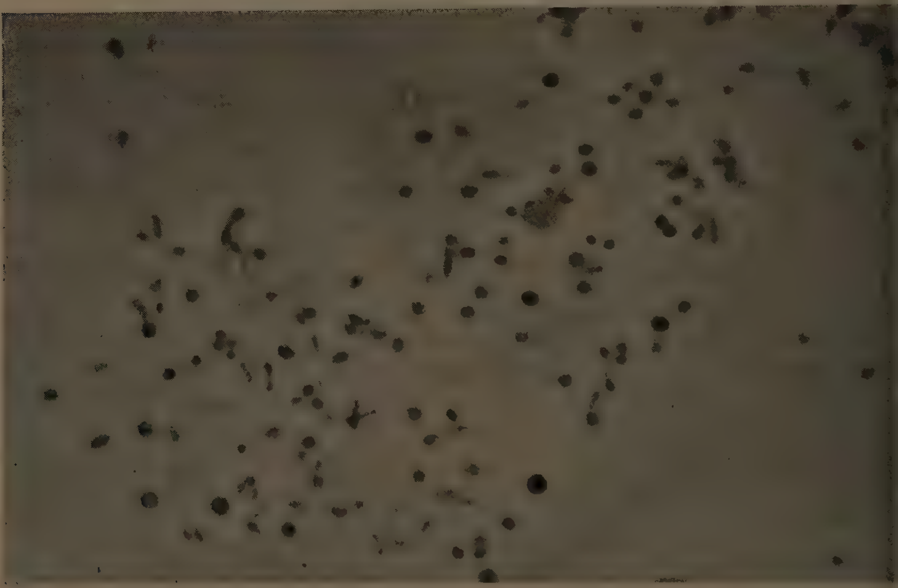

FIGURE 1. Murine PPLO in the fluid of an inoculated HeLa cell culture. Phase contrast, ×1900.

It was hoped that motility would be helpful in identifying the catarrhal PPLO in inoculated HeLa cell cultures. The actual findings were disappointing. Although companion cultures in serum bouillon regularly showed both spinners and gliders, they were rarely observed in the fluid of the tissue cultures. There was also a marked decrease in the number of rods and an increase in the number of spherical forms. In unstained mounts the presence of small particles from degenerating HeLa cells was undoubtedly a source of error in the identification of PPLO. The best microscopic preparation from the fluid of an inoculated HeLa cell culture is shown in FIGURE 1. An occasional glider was observed in this culture during the first day after inoculation.

The Characteristics of a PPLO Contaminant Isolated from Uninoculated HeLa Cell Cultures

It was soon found that the HeLa cell line used in the preceding tests regularly harbored PPLO of unknown source. The study was continued, but now became an assay of two dissimilar organisms in the same medium.

The contaminant was first detected in tube cultures, but particular attention was paid to it in the seed cultures. The fluid in these cultures, containing both penicillin and streptomycin, was changed biweekly. On the seventh day the monolayer was removed with trypsin and the HeLa cells sedimented. Serum-agar plates were regularly inoculated from the cell-free supernatants and, less often, from the washed and resuspended cells. Both inocula yielded colonies of the contaminant on the second day of incubation. The growth was sparse and rarely exceeded twenty-five colonies per microscopic field. It was also recovered from the fluid of the tube cultures and was probably transferred to all of them from the seed suspension. Growth of the contaminant in the fluid of recently established tube cultures was extremely meager and often averaged less than one colony per field. After a series of fluid changes, however, the number of colonies approximated that in the seed cultures. In both cultures the organisms multiplied in the presence of steptomycin sufficient to inhibit growth of the much more vigorous murine PPLO.

The colonies of the contaminant were distinctive on serum-agar plates and usually distinguishable from those of the murine organisms. Three colony types, probably variants of a single species, were recognized. The most common one had a maximum diameter of 400 μ and was barely visible to the naked eye. It showed a delicate internal structure with a network of vacuoles and striations. This area was usually separated from the margin by a clear zone. The two variant colonies were generally smaller, with diameters in the vicinity of 100 μ. One was clear with no internal structure and the other granular with a denser central zone. Colonies of the catarrhal PPLO are uniformly granular, much denser than those of the contaminant, and clearly visible macroscopically. The vacuolated colony of the contaminant is illustrated in FIGURE 2.

Growth of the contaminant in serum bouillon was characterized by a barely visible turbidity that appeared on the second or third day, but only after a number of transfers. Unlike the murine PPLO, the contaminant produced an alkaline reaction with a terminal *p*H of 8.2. On examination with the phase microscope, the fluid cultures showed a sparse distribution of spherical elements, either discrete or in small aggregates. They were nonmotile. Two isolates of the contaminant that had been transferred 10 times in serum bouillon were subcultured on serum agar with and without streptomycin. The organisms were still resistant to the antibiotic and grew as well on one plate as the other.

Several clonal lines of HeLa cells* were cultured on serum agar and yielded PPLO similar to the contaminant isolated from the wild line.

The colonial characteristics of the tissue culture contaminant were surprisingly similar to those of a PPLO isolated some years ago in our laboratory from the nasal washings of sheep with coryza. No relationship between the two types is implied.

* Provided by E. F. Wheelock of The Rockefeller Institute.

Cytopathogenic Changes in HeLa Cell Cultures Inoculated with Murine PPLO

Multiplication of the murine PPLO in HeLa tissue cultures was not attended by any significant change in the cell monolayer during the first three days after inoculation. The cells remained attached to the glass and appeared normal on microscopic examination. The *p*H of the fluid on the third day was about 7.2, and the color had changed from pink to yellow.

Observations were then made on inoculated tissue cultures maintained by the successive renewal of fluid at intervals of 2 to 3 days. With the change of fluid the murine organisms continued to multiply and were demonstrable on subculture to serum agar. In the presence of viable HeLa cells, growth of the organisms was noticeably better than in the fluid alone. Serum-agar plates inoculated on the third day from the fluid of the tissue cultures showed as many as 50 colonies per microscopic field in contrast to 3 or less from the fluid unsupported by HeLa cells.

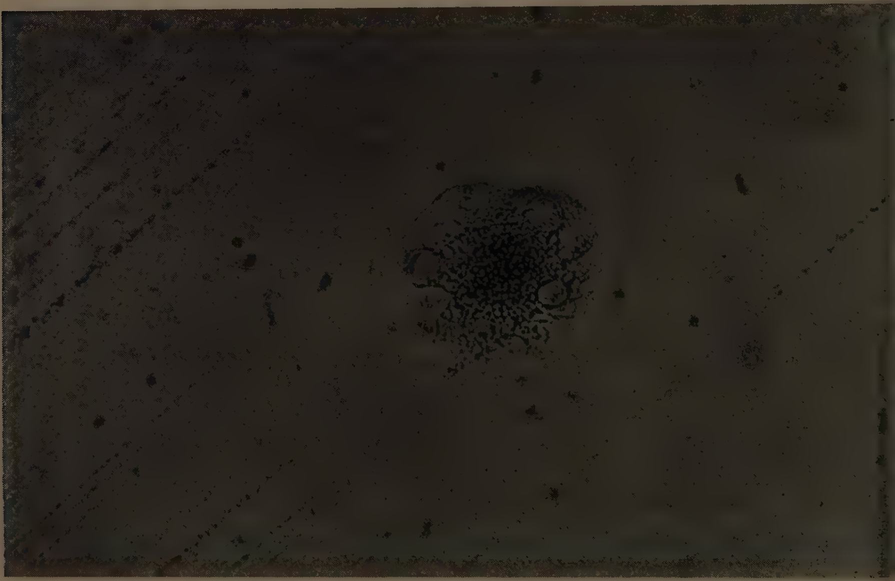

FIGURE 2. Vacuolated colony of the PPLO contaminant from a seed culture of HeLa cells. Serum agar. ×133. Photographed by J. A. Carlile.

After the second or third renewal of fluid, cytopathogenic alteration of the monolayer became apparent. Cells began to appear in the fluid and showed unmistakable evidence of degeneration when examined with the phase microscope. The cell column had a dry powdery appearance and contained discrete granular cells within the network of normal ones. Small groupings of coalesced cells were also present.

This reaction became intensified with subsequent renewals. More cells appeared in the fluid, and the monolayer had a moth-eaten appearance. On microscopic examination, there was an increase in the number of degenerated cells, and the network of normal cells was replaced by scattered individual ones. The aggregates of coalesced cells were increased in size, number, and density. A typical field (×100) is shown in FIGURE 3.

At this time, Wayson-stained films were made of the sediment from the tissue culture fluids. With this stain normal cells are uniformly blue, degenerating ones pink with a blue nucleus, and necrotic ones pink with a pink nucleus. Films of the sediment showed a mixture of all three cell types, with a predominance of necrotic ones. In contrast to this distribution, normal cells predominated in films of the sediment from uninoculated seed cultures.

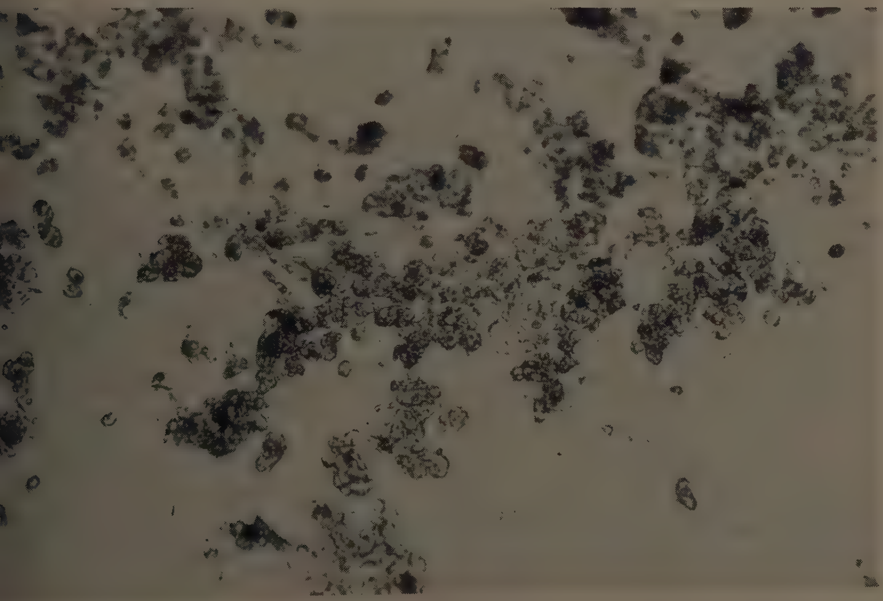

FIGURE 3. Appearance of the monolayer after 3 fluid changes in a HeLa cell culture inoculated with murine PPLO. ×133. Photographed by J. A. Carlile.

With a magnification of 950 diameters, one or more round blue inclusions were sometimes found in the pink cytoplasm of HeLa cells from the inoculated tissue cultures. Other cells showed much smaller blue particles scattered throughout the cytoplasm. Less distinct intracellular bodies were also found in films from the uninoculated but contaminated tissue cultures. These inclusions and particles were present only in degenerating or necrotic cells. They were presumably aggregated or discrete PPLO.

By the twelfth to fourteenth day, after four to five changes of fluid, inactivation of the HeLa cells in the inoculated cultures was virtually complete. In some cultures, groups of the necrotic cells remained attached to the glass. In this event, the color of the fluid changed from pink to yellow, and the murine PPLO continued to multiply. More often, however, all of the HeLa cells disappeared from the glass. The fluid retained the pink color, and no or very few organisms were demonstrable on subculture.

Cytopathogenic Changes in Uninoculated HeLa Cell Cultures

Uninoculated tube cultures of the HeLa cells, similarly maintained by the renewal of fluid, showed slowly progressive cytopathogenic changes. The network of normal cells in the monolayer remained intact for an extended period, and few cells were present in the fluid. Aggregates of coalesced cells eventually appeared in the monolayer. These masses slowly increased in number and some became very dense. The PPLO contaminant was regularly recoverable from the fluids, meanwhile, prior to their renewal.

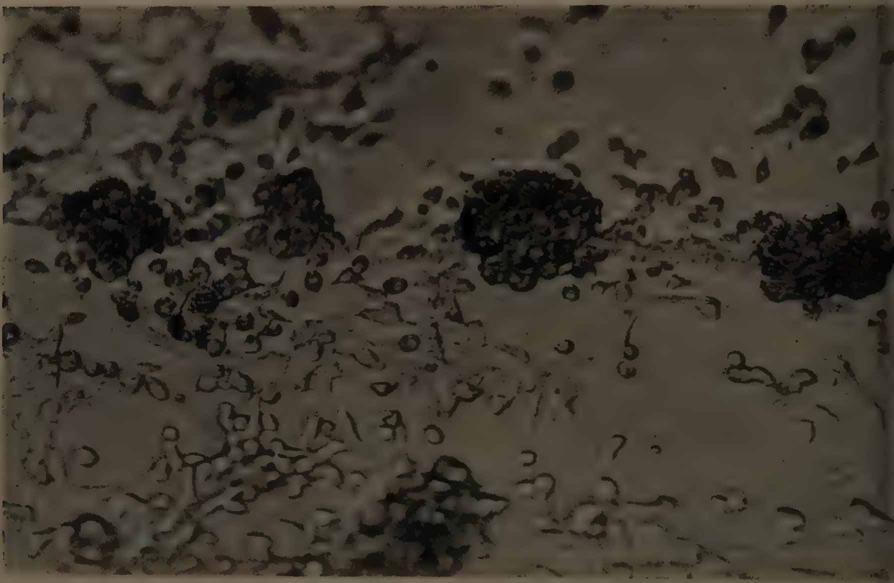

FIGURE 4. Appearance of the monolayer in an uninoculated but contaminated HeLa cell culture after 6 months of continuous incubation. ×133. Photographed by J. A. Carlile.

The HeLa cells in one tube culture, maintained by the biweekly renewal of antibiotic-free fluid, continued to grow for nearly five months in the presence of the contaminant. Near the end of the fifth month, after the monolayer had begun to show very dense cell aggregates, growth suddenly ceased, and the fluid remained pink in color. Two other tube cultures were similarly maintained, with the exception that both penicillin and streptomycin were added to the fluids. Both of these cultures are still alive after seven months of continuous incubation. PPLO are recoverable on subculture, and the fluids change from pink to yellow with each renewal. As indicated in FIGURE 4, the monolayer shows large and dense aggregates with a much reduced network of presumably viable cells.

Comments

The preceding observations obviously suffer from the lack of HeLa cell conrols known to be free from PPLO. It is uncertain whether the accelerated ytopathogenic effect noted with the inoculated cultures is due to the murine PLO alone or to an interaction between them and the contaminant. The ctual cause of the slowly progressing change in the uninoculated cultures is lso uncertain. There is little doubt that both types of PPLO may develop ithin HeLa cells. Whether or not they attack living cells or multiply only dead or senescent cells is a matter of conjecture.

Summary

Murine pleuropneumonialike organisms (PPLO) of the catarrhal type were sed in these experiments. Preliminary tests on their growth in slanted screwapped tubes, containing 1 ml. of medium, indicated that the survival rate was uch lower in tissue culture fluid alone than in 20 per cent horse serum bouillon. he organisms multiplied in HeLa cell cultures and were recoverable by suboculation to serum agar. They were also demonstrable microscopically, but otile forms were rarely observed. Growth was inferior to that in serum ouillon, but was better sustained than in tissue culture fluid alone.

PPLO of unknown source were regularly isolated from uninoculated seed nd tube cultures of the HeLa cells. The contaminant was considerably more esistant to streptomycin than the murine organisms and otherwise sufficiently istinctive to be readily identified.

Successive renewal of the fluid in inoculated HeLa cell cultures was followed y marked cytopathogenic alteration of the monolayer. After 4 to 5 changes ver a period of 12 to 14 days, inactivation of the HeLa cells was virtually omplete. Uninoculated by contaminated tissue cultures showed much slower egenerative changes in the monolayer. In 2 cultures, however, viable cells ere still present after 7 months of continuous incubation.

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DISCUSSION OF PART II

JOHN B. NELSON (*The Rockefeller Institute, New York, N.Y.*): With respect to Madoff's paper, Edward questioned the necessity of using high-power microscopy for examining plates. He felt that a magnification of ×10 with a dissecting microscope was often adequate. He added a word of caution as to the use of impression films and stressed the importance of the medium, particularly the strength of the agar, in colony identification. He noted the value of yeast extract as a growth factor, but pointed out that DNA might also be required to counteract the inhibiting effect of the excess RNA.

In discussing Fabricant's paper, Klieneberger-Nobel observed that she had not worked with chickens, but had studied cultures of avian PPLO isolated by others. She called attention to the long continued work on fowl coryza by H. P. Chu in Cambridge, England, noting that he had identified several different strains of PPLO. Chu referred to the organisms of a strain that caused coryza in chickens and turkeys as coccobacilliform bodies, but regarded them as essentially PPLO. Klieneberger-Nobel did not deny this relationship, but emphasized the distinguishing characteristics of the coccobacilliform bodies.

Smith questioned Fabricant's separation of avian PPLO into distinct serologic and physiological types, because of uncertainty as to whether the cultures studied were type-pure.

Edward stressed the importance of type-pure cultures and suggested the use of an inhibiting antiserum in the culture medium.

Fabricant, in reply, observed that the work of Chu, Adler, and others supported his conclusions concerning the specificity of the S6 type of avian PPLO. He noted that this type was found only in the coccoid form and was serologically distinct. He commented on the difficulties encountered in obtaining type-pure cultures and added that several of his strains of both types were apparently pure by whatever standards were applied.

In discussing Shepard's paper, Klieneberger-Nobel recounted her experiences with a culture of Shepard's adapted T type of PPLO. By employing the results of CF tests, she concluded that it was a chicken coryza strain. She questioned the nature of the colonies shown in certain of Shepard's pictures, pointing out that plates inoculated with exudate and then incubated often contain granular cells that may resemble PPLO colonies. Klieneberger-Nobel also questioned the nature of granules appearing on the surface of cells in stained films of exudate and regarded them as bacteria or bacterial debris.

Dienes noted that the T colonies looked exactly like the earliest stage of L colonies, which grew later to a larger size.

Fabricant stated that his pathogenic avian strains fitted the same description as the T type of PPLO. He advised caution, however, in the interpretation of relationships from morphologic and serologic findings. He was unable under any circumstances to accept the idea that the T type of PPLO were L forms.

Shepard, in reply, stated that his T type had looked the same before and after it was introduced into eggs, and that its relation to avian PPLO was uncertain. He noted that the organisms appearing in and on cells were very inute, less than one third of a micron in diameter if coccoid, and could not be istaken for bacteria. In the known presence of bacteria the particles covering le cells were deeply and intensely stained in contrast to the reaction with lore delicate PPLO.

With respect to Gentry's and Kelton's papers, Smith advised keeping an pen mind concerning a relationship between stable L forms, L forms in general, nd PPLO. He questioned the nature of the starting materials used by Gentry nd Kelton, since they were able to wipe the colonies, together with their enters, from the surfaces of plates. He had repeated the exact procedure utlined by Kelton, using the same medium and the same strains, but starting ith single-colony isolates. Any bacteria (other than PPLO) recovered were lso isolated with equal frequency from uninoculated control tubes.

In commenting on the results obtained by Gentry with his inhibitor and oninhibitor broth, Smith thought that PPLO might lie dormant in the latter nd be overgrown by bacteria.

Landman suggested that methods developed in bacterial genetics might be aluable in connection with the problem of contamination. In dealing with he change from L forms or PPLO to bacteria and back again, it should be ossible to introduce as a marker a sulfonamide-resistant organism or one unelated to nutrition in the bacterial or the subbacterial form. Presence of the arker would distinguish a true derivative from a contaminant.

In commenting on the combined papers of Rothblat, Hayflick, and Nelson, nd in reply to Nelson's comment on the desirability of eliminating the PPLO ontaminant from tissue cultures, Hayflick stated that he had recently devised method for decontaminating PPLO or L-form infected cell lines. This ethod depended on the differential heat lability of PPLO and tissue culture ells. Hayflick said that he was able to decontaminate 2 infected cell lines by cubating them at 40° C. for from 12 to 18 hours. These cultures, on subseuent platings, remained free from PPLO for 1 month. The hyperthermic reatment was not without some effect on the tissue culture cells. HeLa cells t 40° C. had a tendency to arborize, with some rounding and cytoplasmic vaculization. These changes were not fatal, and recovery was usually assured on educing the temperature to 37° C. Confirmation of the heat lability of PPLO, Iayflick said, was obtained by incubating inoculated agar plates at 40° C. for 2 hours. The treatment resulted in a total loss of viability. The preheating f tissue culture fluids at 50° C. for 30 min. was also suggested. Hayflick mphasized the preliminary nature of these results.

An unidentified speaker asked to what extent the presence of PPLO repreented a problem in terms of cell growth and virus multiplication.

Edward asked if any attempt had been made to identify the PPLO recovered rom tissue cultures. He noted that *Mycoplasma hominis* had been reported n some instances by other workers. Two PPLO cultures isolated from cell ines maintained in his laboratory were unidentified, while a third culture proved o be an avian organism similar to the S6 type. Edward expressed an interest n the origin of the tissue culture contaminants.

Nelson doubted that the contaminants were introduced by the serum or the nedia constituents and ventured the guess that PPLO were present in some cell lines from their beginning. Hayflick objected to the latter intimation and reported that two lines of HeLa cells that he had used prior to the reports of Collier and of Robinson *et al.* were free of PPLO.

Nelson then observed that a single subculture, or even several negative ones, did not constitute proof that a given cell line was uncontaminated, and cited his experience with the use of Aureomycin. A seed culture of HeLa cells known to be contaminated with PPLO was maintained for ten weeks, with biweekly fluid changes, in the presence of a nontoxic low concentration of the antibiotic. During this period serum-agar plates subcultured from both the cells and the fluid were regularly sterile. The seed culture was then maintained in the absence of Aureomycin. After a single change of fluid, PPLO identical with the original contaminant reappeared and were regularly demonstrable on subculture.

Nelson likened the employment of contaminated tissue cultures to the use of naturally infected experimental animals. In both instances, he said, the observed response to an introduced agent might be abnormal and might lead to false conclusions. The possibility of an interaction between the PPLO contaminant and a virus was indicated by his findings with a neurotropic strain of mouse hepatitis virus. Intracerebral injection of a mixture containing the virus and PPLO isolated from HeLa cells was attended by a pathological reaction unlike that produced by either component alone.

art III. Classification of PPLO

RELATION OF PPLO TO BACTERIA*

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The identification of pleuropneumonialike organisms (PPLO) as a distinct roup of microorganisms is dependent upon resolution of the relationship of iese organisms to stable L forms and bacteria. Prior to the use of inorganic lts as an adjunct to the production and isolation of L forms, the only criterion ifferentiating stable L forms from PPLO was the knowledge that the L form as derived from a bacterium. Thus, without this knowledge, those stable forms not requiring a hypertonic environment for survival and reproduction re indistinguishable from PPLO. Although this is not direct evidence that PLO are stable L forms, it does cast doubt on the premise that PPLO are a istinct group. Classification of these organisms should be postponed until iis prime issue is resolved.

We have been conducting studies relative to the possible conversion of the ampo strain of PPLO to diphtheroids and the production of L forms from iese diphtheroids. As previously reported,[1] diphtheroids serologically cross-eacting with the Campo PPLO could be isolated only in large-volume broth ultures and then only in a frequency as high as once in 10^{10} to 10^{11} PPLO cells hen 5 per cent yeast extract was present in the medium. This phenomenon as been observed repeatedly. Some attempts have been made to increase the equency of diphtheroid appearance, but such experimentation necessitates a ather thorough identification of the resulting diphtheroids. Included in these ttempts were the addition of various fractions of diphtheroids isolated from ampo cultures. Thus, heat-killed diphtheroids, the phenol-insoluble residue r cell wall obtained by sonication of the diphtheroids, the acid-hydrolyzed ell walls of the diphtheroids, and the crude desoxypentose nucleic acid fractions f the diphtheroids were incorporated into the medium inoculated with the ampo PPLO. Uninoculated controls were likewise run. In no instance was he frequency of diphtheroid appearance elevated above the control containing one of these fractions. Lowering the oxidation-reduction potential of the iedium by physical or chemical means and incorporating varying mixtures of nown compounds making up the cell walls of bacteria also had no effect. ractionation of yeast extract for the purpose of isolating some factor active this conversion of PPLO to diphtheroids has not been attempted yet.

At this point it is opportune to describe briefly the two types of diphtheroids solated from Campo cultures. Both types give slightly raised colonies about mm. or less in diameter on extract agar. One type, D-17, gives a Gram-ariable staining reaction, and consists of typical club-shaped organisms about

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1 μ in length and of large round forms about 1 μ in diameter. The other type, D-5, exhibits homogeneously small club-shaped rods lying in cuneiform masses. The D-5 grows more luxuriantly than the D-17.

Numerous unsuccessful attempts have been made to derive L forms from the diphtheroids isolated from cultures of the Campo PPLO. However, Dienes was able to produce with penicillin and salts an L form from one of two diphtheroid isolates given to him. This L form derived from the D-5 diphtheroid and designated D-5-L requires at least 2 per cent salt in the medium, but does not require serum for growth. It grows well on agar medium in typical "fried-egg" colonies, but fails to reproduce in broth. The inability to propagate this L form in broth has prevented the production of an antigen for serologic studies. Presently we are engaged in experiments designed to produce L forms more typical of PPLO from both types of diphtheroids utilizing HeLa tissue cultures as the transformation medium. Preliminary experiments have indicated that this method might prove successful.

TABLE 1

COMPARISON OF THE COMPOSITION AND THE GROWTH REQUIREMENTS OF PPLO AND DIPHTHEROIDS

	PPLO			Diphtheroids		L form
	07	Campo	Laidlaw B	D-17	D-5	D-5-L
Sterol	+	+	0	0	0	N.D.
Nature of sterol	cholesterol	cholesterol	ether soluble yellow pigment	—	—	N.D.
Diaminopimelic acid	0	0	0	trace	+	N.D.
Lipoprotein requirement	+	+	0	0	0	0
Salt requirement	0	0	0	0	0	+

To prove a direct relationship of PPLO to bacteria is difficult. Hence, any one type of data by itself is insufficient. Therefore, several approaches have been followed, namely comparisons of the chemical composition, the physiological reactions, and serologic cross reactions of both PPLO and the diphtheroids.

Two unique substances were of particular interest in regard to the chemical composition of the two types of organisms. The "parasitic," PPLO contain cholesterol, while many Gram-positive bacteria contain diaminopimelic acid (DAP) in their cell walls. The PPLO, being devoid of a cell wall, should contain no DAP. TABLE 1 shows that the parasitic PPLO contain cholesterol but no DAP, while the reverse is true for the diphtheroids. Of particular interest is the presence of only a trace of DAP in D-17, the diphtheroid with the bizarre morphology. This diphtheroid could represent an intermediate between the Campo PPLO and D-5, but presently such a relationship is only a result of conjecture. The absence of data on the L form of D-5 is the result of the inability to grow large quantities of this form. In regard to the growth requirements of these various forms, only the "parasitic" PPLO possess a requirement for the lipoprotein and only the L form a requirement for salt.

TABLE 2 compares the various distinguishing enzymatic activities of PPLO and diphtheroids. All strains possess a lipase capable of splitting neutral fats nd, in all cases, it is located at the cell surface. The "parasitic" PPLO contains an active cholesterol esterase. The saprophytic PPLO possesses weaker sterase activity. Of particular interest is the presence of some esterase activity in the D-17, but not the D-5 diphtheroid. This finding parallels the difference in DAP content of these microorganisms.

The parasitic PPLO under consideration are capable of oxidizing short-chained fatty acids, but incapable of oxidizing glucose. The diphtheroids D-5 and D-17 associated with the Campo PPLO also are capable of oxidizing fatty acids, but do take up small amounts of O_2 in the presence of glucose as a substrate.

TABLE 2

COMPARATIVE ENZYMATIC ACTIVITIES OF PPLO AND DIPHTHEROIDS

Activity	PPLO			Diphtheroids	
	O7	Campo	Laidlaw B	D-17	D-5
Cholesterol esterase	+	+	±	±	0
Lipase	+	+	+	+	+
Fatty acid oxidation	+	+	0	+	+
Glucose oxidation	0	0	+	±	±

TABLE 3

CROSS-AGGLUTINATION REACTIONS OF PPLO AND DIPHTHEROIDS

Antiserum	Antigen				
	Campo	D-5	D-17	O7	T-5
Campo	2048	0	2	256	8
D-5	64	1024	4	16	4
D-17	32	4	4096	8	4
O7	128	0	0	256	0

TABLE 4

CROSS-PRECIPITATION REACTIONS OF PPLO AND DIPHTHEROIDS

Antiserum	Antigen		
	Campo	D-5	O7
Campo	64	8	256
D-5	2	128	32
D-17	8	64	32
O7	64	64	256

A serologic examination of the different organisms is being conducted. TABLE 3 lists the results of cross-agglutination reactions. Employing the Campo PPLO as antigen, significant cross agglutination occurred not only with the O7 PPLO but also with the two diphtheroids. Aside from the cross agglutination of the O7 antigen by the Campo antiserum, no other significant cross reactions were noted. One PPLO isolated from tissue cultures failed to cross-react with either the other PPLO strains or the diphtheroids. The failure of the diphtheroid antigens to react with the antisera of the Campo PPLO and of each other might be explained by a masking of diphtheroid antigens common to the Campo PPLO. Thus, using whole cells of diphtheroids that possess cell walls, a cross reaction with antiserum of the Campo PPLO, which does not possess a cell wall, would not be expected. A means to eliminate this postulated masking effect would be the use of precipitation tests with solubilized antigens. The results of the cross-precipitation tests are incomplete. However, the data thus far obtained (TABLE 4) indicate that removal of the cell membrane of the Campo PPLO by sonic lysis and centrifugation removes the more reactive cross-agglutinating antigens. On the other hand, removal of the cell wall of the D-5 diphtheroid by disruption in a Nossel shaker followed by centrifugation releases antigen that cross-reacts with both PPLO and the D-17 diphtheroid antisera. Since in these precipitation tests antigen was diluted instead of antiserum, the titers are not as significant as agglutinin titers.

The results of this study are incomplete. Nevertheless, the accumulated data warrant some speculative conclusions. If it were postulated that the D-5 diphtheroid is the parent bacterium, the D-17 an intermediate form, and the Campo PPLO the stable L form, it is reasonable to anticipate the presence of the graded amounts of DAP found, the correlation between the lipoprotein growth requirement, the presence of sterol and cholesterol esterase activity, and the omnipresence of fatty acid oxidative activity. On morphologic grounds, there is also a gradation from a typical diphtheroid (D-5), to a diphtheroid containing what might be considered "large round bodies," so often described during L-form transformations, to a form indistinguishable from PPLO.

Serologic tests have shown a cross reaction in instances where the possible interference of the cell wall of the diphtheroids has been eliminated. All these data tend to suggest that the Campo PPLO may be a stable L form of a diphtheroid.

Needless to say, such a conclusion, although tentative, is subject to doubt. Any final conclusion must await demonstration of the reverse phenomenon, that is, production of a stable L form identical to the Campo PPLO in antigenic composition and physiological activity. Indeed, to say that all PPLO are stable L forms requires demonstration of a direct relationship between many strains of PPLO and bacteria.

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REVERSION OF AVIAN PLEUROPNEUMONIALIKE ORGANISMS TO BACTERIA

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Introduction

It is already known that L forms are derived from bacteria.[1] Pleuropneu- onialike organisms (PPLO), however, are considered a distinct group and e classified accordingly. The isolation of bacteria from PPLO has been re- orted by Minck,[2] McKay and Taylor,[3] Peoples *et al.*,[4] Wittler, *et al.*,[5] and cently by Smith *et al.*[6] With the exception of Smith, these authors describe oservations rather than experiments conducted to determine the relationship L forms to PPLO. The experiments described below were designed to examine ases of microbial dissociation in the hope of throwing some light on the re- tionships among PPLO, L forms, and bacteria.

For purposes of introductory clarification it is advisable to consider briefly e bacterium *Hemophilus gallinarum* and the avian PPLO *Mycoplasma galli- rum* which, we believe, can revert spontaneously to the bacterium or be in- iced to do so.

The literature of microbial dissociation has been reviewed extensively by adley.[7,8] Specific reference to dissociation of the *Hemophilus* species is made in e works of Deacon and his associates[9] and of Smith.[10] Our observations on e morphologic and colonial characteristics of this genus are essentially milar to, but less extensive than, the observations of these workers.

Hemophilus gallinarum

H. gallinarum was first isolated in Europe by DeBlieck[11] and later in the nited States by Nelson.[12] The species is characterized by wide variation oth in morphology of the individual organisms and in colonial types. Typi- lly in its smooth (S) phase, *H. gallinarum* is a Gram-negative bipolar cocco- cillus, but every gradation between this type and the atypical long filaments the rough-(R-)type colonies is encountered. The rods may be thick, plump, rved, segmented, or septate, and may possess metachromatic granules of the treme R phase. The Gram-stain reaction varies from negative in the S type variable or positive in the R types. The morphologically typical and atypi- l bacilli show a corresponding difference in colonial form. The typical bacilli ve smooth colonies with a butyrous consistency, while the atypical tend to ve rough colonies with a dry friable consistency. The S-type colonies are nvex, shiny, and dome-shaped with a smoothly curved edge, while the R pes have a roughed surface and a sharp angular edge. Between these ex- emes there are many transitional or intermediary types. These changes re- lt from mutations, and the new forms are, in general, as stable as the ones om which they were derived.

Several strains have been isolated in our Poultry Pathology Laboratory from ecimens submitted for diagnostic purposes. The strain selected for use in ese experiments was isolated from a white Leghorn pullet with infectious coryza. Initial isolation on blood-yeast-agar plates (10 per cent of each) yielded colonies that, after 24 hours' incubation, were transparent, convex, and shiny, with a diameter of 0.5 to 1.0 mm. After 48 hours the centers were often slightly raised, producing an umbonate appearance. Luxuriant growth of this strain was obtained on medium containing beef-heart infusion broth plus coenzyme 1 at a concentration of 2.5 μg. per ml. It was dependent upon the V factor but not the X factor, and exhibited satellitism when grown adjacent to staphylococci. Biochemically this strain fermented maltose, dextrose, and sucrose, but not lactose, xylose, dulcitol, mannitol, raffinose, arabinose, or glycerol.

Mycoplasma gallinarum

Delaplane and Stuart[13] isolated a PPLO from chronic respiratory disease (CRD) of chickens and suggested that it was identical with a coccobacilliform organism that Nelson[12] had isolated earlier from an upper respiratory infection of a fowl. More recently Adler,[14] Markham and Wong,[15] and Grumbles *et al.*[1 have accepted this PPLO as the agent of CRD. The morphologic, biochemical cultural, and serologic properties of the organism have been studied intensively principally by Adler and Yamamoto[17] and Gianforte *et al.*[18] Four strains of PPLO that reverted to *H. gallinarum* in our experiment were supplied by E. L. Jungherr of Storrs, Conn. and are designated here as J1, J2, J3, and J4; were provided by Julius Fabricant of Cornell University, Ithaca, N.Y., and designated C6, C7, C9, C16, and C18; 1 was provided by A. S. Greig of the Animal Disease Research Institute, Hull, P. Q., Canada, and designated V1; and strains, V2, V32, V36, and 1499, were isolated in our Poultry Diagnostic Laboratory.

The experiments performed with these strains are classified according to whether reversion to *H. gallinarum* occurred spontaneously or was induced experimentally. Although we report only those experiments in which reversion occurred, it must not be implied that reversion can be produced at will—the failures to cause reversion far outnumber the successes.

Spontaneous Reversion of Avian PPLO to H. gallinarum

Spontaneous reversion of two strains of avian PPLO to *H. gallinarum* ha already been reported by McKay and Taylor.[3] The experiments described here record the spontaneous reversion of 6 additional strains, 1499, C6, C7 C9, C16, and C18. The history of strain 1499 will be given in detail; that o the other strains will be given briefly.

Strain 1499 was isolated from a case of CRD in 1955 and, since it was considered as a biochemically and cytologically typical PPLO at that time, it wa maintained as a stock strain by passage through the yolk sac of embryonated eggs at 5-day intervals. At each passage yolk-sac material was cultured on PPLO agar with 1 percent of bovine-serum fraction added, and on to sheep blood agar (10 per cent) that had been streaked with staphylococci, and incubated at 37° C. Each passage and each plating were made with 0.1 ml. o harvested yolk-sac material. All yolk-sac residue was preserved at −20° C

All passages up to No. 55 gave PPLO alone when cultured on solid media

Egg passage No. 56 gave *H. gallinarum* after 5 days' incubation. The organism was in the S phase and exhibited typical satellitism. Growth from the solid medium was subjected to 11 additional passages through the yolk sac and eplated at each passage. From each replating *H. gallinarum* alone was recovered.

The yolk sac residue of the fifty-fifth passage, which had been frozen for 7 days, was passaged 3 times more through eggs. From the first 2 of these passages no growth of any kind was obtained on solid media; from the third-passage material *H. gallinarum* grew. We repeated this with the residue of the fifty-fourth passage, which had been frozen for 14 days, passing it 4 times through eggs. From the first 3 of these passages there was no growth on the solid media but, from the fourth, *H. gallinarum* was obtained. Intermediate forms were not observed.

The C strains were received in frozen yolk. This material, plated on solid media, yielded PPLO but no *Hemophilus*. Strain C6 was passaged twice through the yolk sac of embryonated eggs. Culture on solid media from the irst passage yielded a Gram-variable rod and from the second passage *H. gallinarum* in S phase. The remaining C strains, 9, 16, and 18, yielded the same Gram-variable rod from each 2 yolk-sac passages. These strains were submitted to a further series of yolk-sac passages, and the S-phase *H. gallinarum* was obtained on solid media after the third passage.

The Gram-variable bacilli recovered in the intermediate stage of these reversions are considered in more detail later.

Induced Reversion of Avian PPLO to H. gallinarum

The acute form of infectious coryza of chickens is attributed to *H. gallinarum*.[19] The PPLO isolated from the chronic form of this disease is *M. gallinarum*, previously described by Nelson[12] as coccobacilliform bodies. Since PPLO have been incriminated in the etiology of CRD, it is suggested that avian CRD might be merely a chronic expression of infectious coryza. This suggestion implies that the PPLO may have been but L forms of *H. gallinarum* and that, under appropriate laboratory conditions, they may revert to *H. gallinarum*. Griffith[20] in 1928 demonstrated the interconvertibility of S and R strains of the pneumococcus by incubating one strain with a heated extract of the other. We performed this type of experiment with our organisms, first using saline extracts of heat-killed *H. gallinarum* and, later, a crude extract containing DNA rom the bacterium.

Reversion Induced by Saline Extracts of Heat-Killed H. gallinarum

Methods. The method of preparing the extract of *H. gallinarum* was that used by Griffith[20] working with the pneumococcus. *H. gallinarum* was cultured on blood agar plus yeast extract (10 per cent), and the young S colonies obtained after 18 to 24 hours of incubation were washed off in physiological saline. The washings were pipetted into 20-ml. vaccine vials, immersed in a water bath at 70° C. for 30 min., centrifuged in an angle centrifuge at 1500 rpm. for 15 min., and the supernatant decanted and cooled. If smears of the supernatant revealed the presence of intact organisms the process was repeated. Occasional difficulty in lysing the cells was overcome by adding 1.0 ml. of a 10 per cent solution of sodium desoxycholate to 100 ml. of the culture washings one-half hour before heating.

The strains of PPLO used, J1, J2, J3, and J4, were obtained in broth. These were cultured first on solid media to ensure that no *H. gallinarum* was present. We then inoculated the yolk sac of 7-day embryonated eggs with 0.1 ml. of the broth culture of each strain of PPLO to which had been added 0.1 ml. of the extract of the bacterium 30 min. before inoculation. As a control we inoculated 2 other series of eggs with, respectively, 0.2 ml. of the heated extract of *H. gallinarum* and 0.2 ml. of broth culture of the PPLO, and maintained a third series of uninoculated eggs as batch controls. The eggs were candled daily and harvested when they showed signs of abnormality. The harvested yolk material was examined on direct smear for bacilli and by culture on solid media. The plates were examined daily for 5 to 7 days with the dissecting microscope and subcultured by the agar-block technique, as for PPLO, every 2 days.

TABLE 1
REVERSION OF J STRAINS

Number of strain		Number of transfers on solid media	Reversion to bacillary phase
J1	PPLO culture	5	None
J2			
J3			
J4			
J1	PPLO culture plus heated culture of *H. gallinarum*	3	All strains
J2		4	
J3		4	
J4		3	
Control No. 1: heated culture of *H. gallinarum*		5	None
Control No. 2: medium		5	None

Results. These are summarized in TABLE 1. The eggs that were uninoculated or inoculated with the heated extract of *H. gallinarum* alone were subcultured 5 times on solid media and yielded no growth. The eggs that had been inoculated with a broth suspension of PPLO yielded these organisms but no bacilli on each of 5 subcultures. However eggs that had been inoculated with the heated extract of *H. gallinarum* mixed with a suspension of PPLO yielded colonies of bacilli on the third subculture in the case of strains J1 and J4 and, on the fourth subculture, in the case of strains J2 and J3. The bacilli derived from J1 and J4 were the typical smooth, Gram-negative form of *H. gallinarum* exhibiting satellitism, but the bacilli obtained from J2 and J3 were Gram-variable and produced R colonies. These Gram variants will be referred to again when dissociative phases are described. We repeated these experiments on two subsequent occasions, having meanwhile maintained the PPLO by yolk-sac passage. The only difference between the results of the first and repeated experiments was that, in the latter, the bacilli obtained were n each case Gram-variable and produced R colonies, which were reverted to the S phase.

Reversion Induced by Crude DNA Extracts of H. gallinarum

Methods. Three types of artifical media were employed: beef-heart infusion broth (Difco) to which 1 per cent serum fraction (Difco) and coenzyme 1 at a concentration of 2.5 μg./ml. were added just before inoculation (medium A); nutrient agar plus 10 per cent sheep blood streaked with staphylococci (medium B); and PPLO agar (Difco) with 1 per cent serum fraction (Difco) and thallium acetate added to a concentration of 1/1000 (medium C).

The methods of Alexander and Leidy,[21] McCarty and Avery,[22] and Mayers and Spizizen[23] were modified to prepare the DNA in the following manner. A 10-ml. overnight culture of *H. gallinarum* in the medium A was inoculated into 12 liters of the same medium. The culture was grown for 10 hours, and centrifuged in a Sharples supercentrifuge at a maximum speed of 25,000 rpm. and a rate of flow of 60 ml./min. The sedimented organisms were suspended in 150 ml. of a 1 per cent aqueous solution of sodium lauryl sulphate and bile salts, to which was added 150 ml. of a saturated solution of sodium acetate. The *p*H of these reagents was adjusted to 7.0 prior to use. This suspension was heated and stirred constantly in a water bath at 60° C. for 15 min. The remainder of the procedure was carried out at 5° C. The material was centrifuged for 90 min. at 1500 rpm. and the clear supernatant liquid decanted into 2.5 volumes of ethanol cooled to 5° C.; the mixture was then agitated by rotation. Strings of nucleic acid precipitated out and were removed from the solution on a glass rod or by filtration for suspension in 100 ml. of a 0.14 M solution of sodium chloride and storage at 5° C.

The yield of DNA fraction from 12 liters of broth cultures was found to vary from 6 to 8 mg. The DNA fraction dissolved slowly in 0.14 M sodium chloride and dissolved readily in 0.6 M sodium chloride. Upon precipitation in alcohol, it was viscous. Attempts were made to purify it by removing the polysaccharides and ribonucleic acids, but these procedures resulted in a loss of activity. It was necessary to prepare fresh material for each series of experiments. Not all fresh preparations contained the active principle.

Sperm DNA* and calf-thymus DNA, the latter prepared by G. Butler of the University of Toronto, Toronto, Canada, were used in the control experiments to check the specificity of the bacterial DNA. The results obtained with these DNA fractions were negative, and they will not be considered further.

The strains of PPLO used in these experiments were V1, V2, V32, and V36. The methods of handling and the results obtained are given in detail for strain V1 and subsequently we shall point out any difference with respect to the other strains. Strain V1 was subjected to 10 serial passages, at 4-day intervals, through the allantoic cavity of 9-day embryonated eggs and, at each passage, was cultured on the solid media B and C to examine for stability of type as a pleuropneumonialike organism.

Tenth-passage allantoic fluid was inoculated into medium A and, after 21 hours of incubation, was subcultured in the same medium for 3 more hours, when 0.3 ml. of the DNA-containing extract, at a concentration of 18 μg./ml., was added. Control tubes of medium plus DNA extract were run in parallel. These mixtures were allowed to incubate; at 3-, 12-, and 72-hour intervals, 0.2-ml. aliquots were inoculated into the allantoic cavity of 9-day embryonated eggs and, at the same time, 0.1 ml. of the same material was plated on solid media B and C. Four serial passages at 3-day intervals were made via the allantoic cavity, and material from each passage was plated on the solid media B and C. The plates were examined daily for 7 days for growth of bacilli and PPLO. Additional controls were prepared by passaging the solution of DNA serially for 4 passages via the allantoic route and culturing on solid media from each passage, including uninoculated eggs as a control to ensure sterility. These trials were repeated 30 times, but positive results were obtained in only 5.

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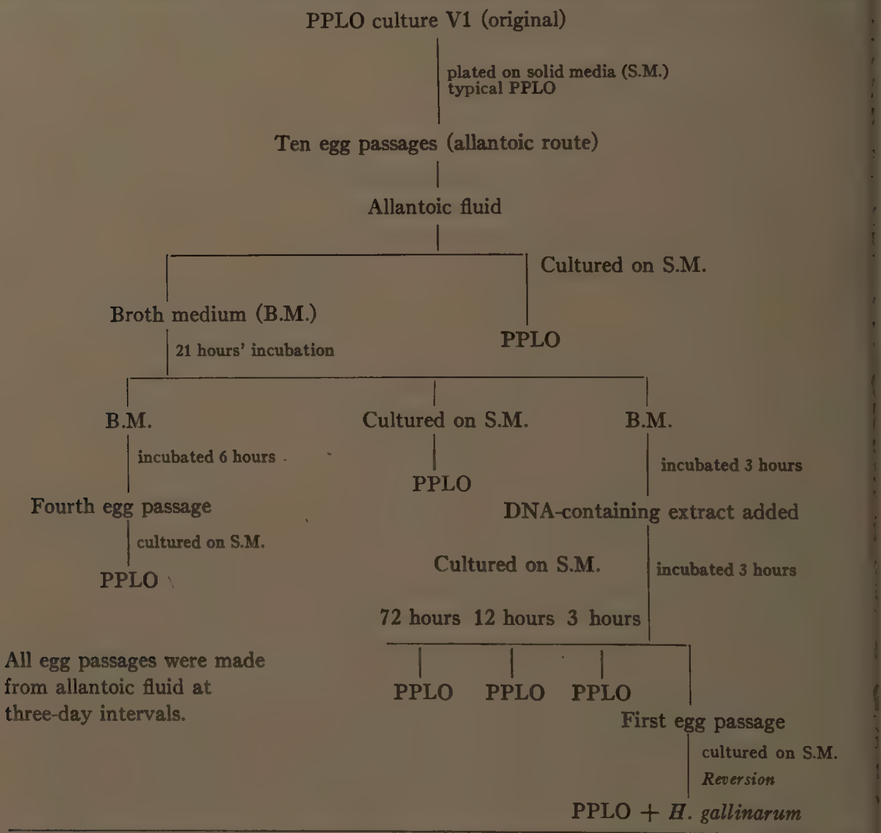
* Obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE 2

METHOD OF REVERSION OF PPLO STRAIN V1 (HULL)



Results. The results obtained with strain V1 are summarized in TABLE 2.

FIGURE 1*a* to *c*, photographs of strain V1, show the PPLO in the original broth culture. They demonstrate the presence of L bodies with intracellular elementary corpuscles (FIGURE 1*a*); *b* and *c* show an L body extruding a pseudopodial ending. Gram-stained smears of the broth did not reveal bacillary forms. Colonies of strain J1 showing L bodies are illustrated in FIGURES 5*d*, 6*a* and *b*.

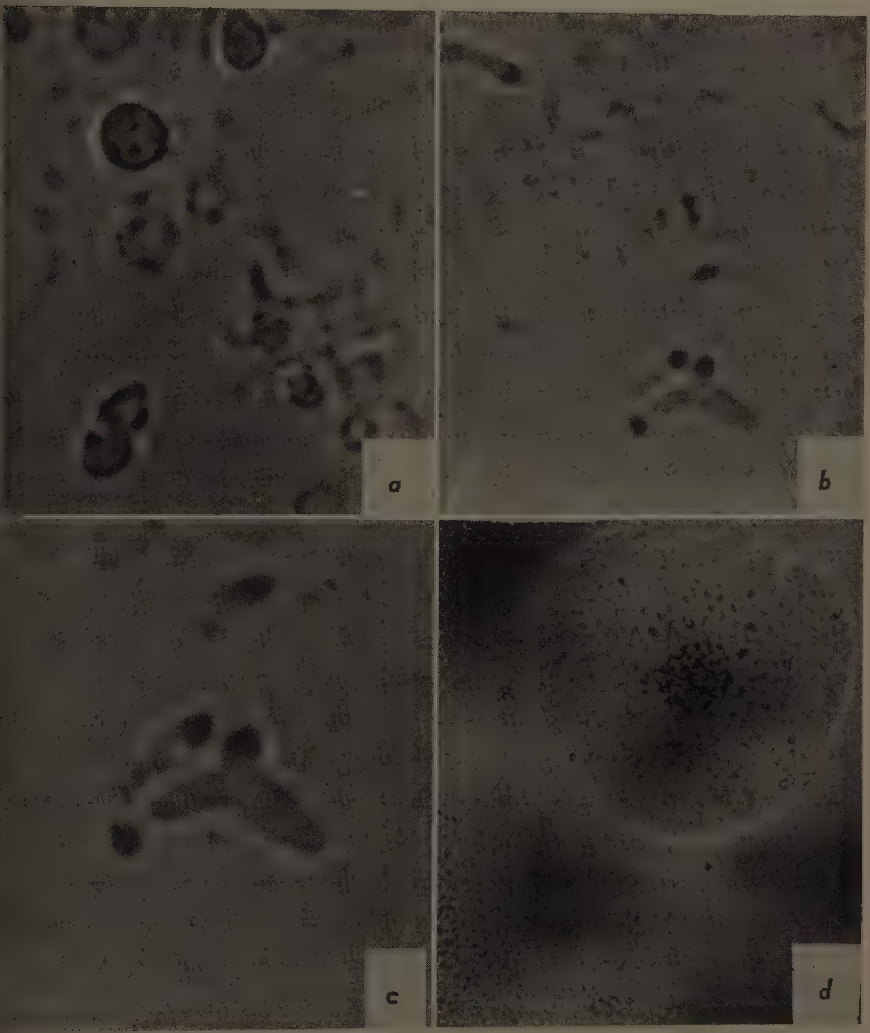


FIGURE 1. Reversion of PPLO strain V1 (Hull). (*a*) L bodies show intracellular elementary corpuscles in the broth culture. ×4500. (*b*) L body extruding a pseudopodial ending. ×3000. (*c*) Selected from *b*. ×4500. (*d*) PPLO colony incubated 4 days on solid medium. Isolated from the broth culture. ×400.

Colonies of strain V1 are shown in FIGURE 1*d* as they appeared following 4 days' incubation on the solid media inoculated with first-passage allantoic fluid. Bacterial forms were not observed on Gram-stained smears or in wet mounts of the growth. Figure 2*a* shows an L body with elementary corpuscles arranged about the periphery, while the adjacent L body appears to be devoid of elementary corpuscles. Figure 2*b* demonstrates a young colony composed entirely of L bodies.

After 10 egg-to-egg passages, Gram-stained smears and wet mounts of the growth of strain V1 on the solid media showed that the PPLO had retained their original form; the bacillary phase was not evident.

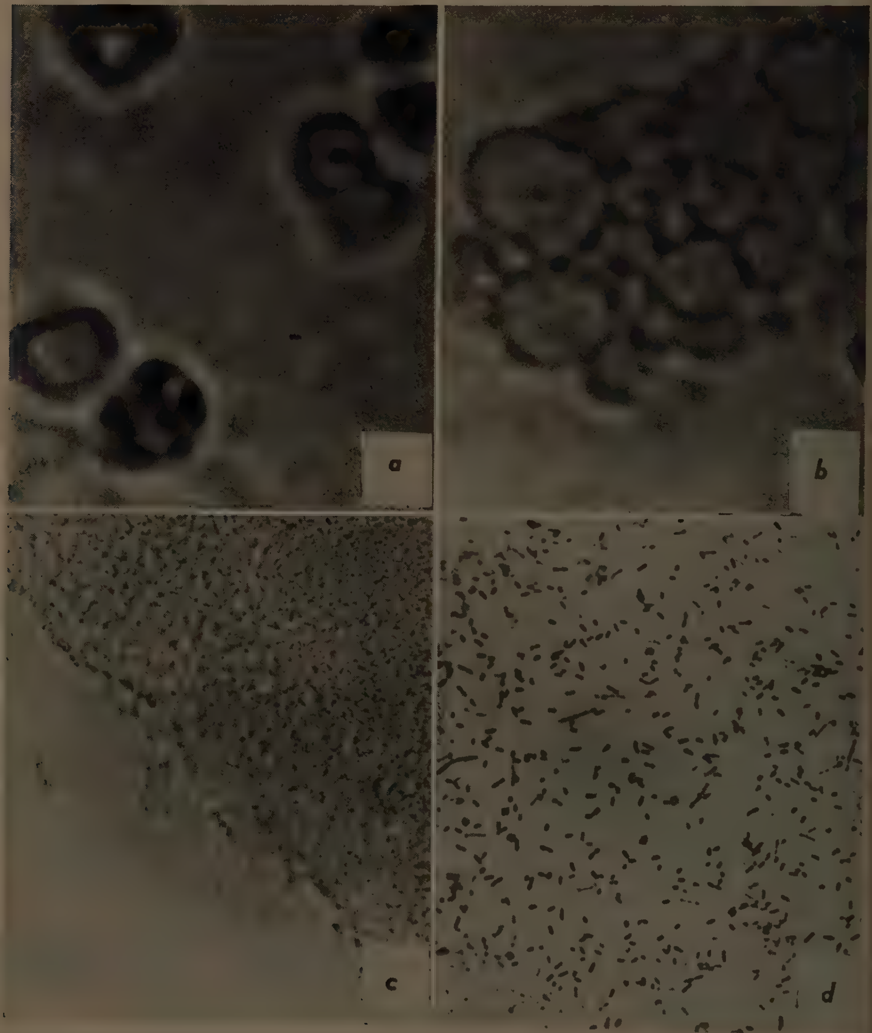


Figure 2. Reversion of PPLO strain V1 (Hull). (*a*) Two adjacent L bodies, one vacuolated and the other showing elementary corpuscles arranged about the periphery. ×4500. (*b*) A young colony composed entirely of L bodies. ×3000. (*c*) Small colony containing minute granules, from agar plate figure 3*b*. ×1000. (*d*) Gram-negative bipolar rods converted from the PPLO phase of strain V1. Large smooth colonies showing satellitism (figure 3*b*). ×1000.

Cultures made at 3-, 12-, and 72-hours' incubation periods of the broth containing PPLO and DNA extract yielded only typical PPL colonies, reversion not occurring *in vitro*. The control series was negative. Allantoic fluid from each of the first 3 egg passages of the PPLO culture to which DNA extract had been added showed the following results when cultured on solid media: (1) PPLO colonies on medium C; (2) on medium B a mixture of small colonies containing minute granules (FIGURE 2*c*) and colonies that were smooth and convex, consisting entirely of Gram-negative rods (FIGURE 2*d*) and showing satellitism (FIGURE 3*a*). Subculture by the agar-block technique onto the same medium yielded the same colonial types, but the growth was more luxuriant (FIGURE 3*b*). Strains V2 and V32 reverted to bacillary form in the third egg passage, and V36 reverted in the fourth passage.

The bacillus recovered from the PPLO in these experiments was in the S phase and Gram-negative. It grew only in the presence of the V factor or as a satellite with staphylococci. The organism fermented the same sugars as previously described.

The DNA fraction used in these experiments was a crude one. The method of Zamenoff *et al.*[24] was used to remove the protein from the crude extract of DNA, and the deproteinized material was employed, in place of the crude extract, in the same experimental pattern as above. The results were negative.

Colonial and Morphologic Variants of H. gallinarum

We have already alluded to Gram-variable bacilli as variants of *H. gallinarum* in the experiments cited above, in which we effected reversion of the avian PPLO strains J and C to the bacterium. We have not made an intense study of these intermediate variants, but we have recorded a few observations on the manner in which these variants may be produced from the S phase of the bacterium and on the manner in which they may be reverted to the S phase. When these reversions can be effected, it is not difficult to show that the final bacillus is *H. gallinarum* and to accept the intermediates as variants of this organism. It should be pointed out however, that we have recovered this variable bacillus by yolk-sac passage of other isolates of avian PPLO without being able to effect any further alteration toward a typical *Hemophilus*.

If S-type colonies of *H. gallinarum*, the smoothest being obtained after passage through the yolk sac, are allowed to age at room temperature on plates of sheep-blood agar streaked with staphylococci, there are progressive alterations in the morphology of the colonies and of the organisms. The S colonies show satellite growth, with the largest colonies growing in the neighborhood of the staphylococcus streak. They are shiny; some are flat and tend to be confluent; others are more convex. The flattened colonies have irregular outlines, and the convex colonies are entire. We consider the convex to be the S type, while the flat are intermediate R types (FIGURE 3*c*). The organisms are small Gram-negative coccobacilli (FIGURE 3*d*). After yolk passage the smoothest S colonies are convex, entire, shiny, and umbonated. After 48 hours of incubation the colonies enlarge and become slightly granular and opaque (FIGURE 4*a*). The bacilli are Gram-variable, bipolar or septate, longer, and thicker (FIGURE 4*b*). This R type was recovered from strains J1 and J4, and the C strains after they had been passaged in eggs and cultured on sheep-blood agar. With further aging at room temperature, the R colonies enlarge and differentiate into a central portion with a granular and contoured surface, an intermediate flattened portion, and a beveled irregular periphery (FIGURE 4*c*). The bacilli are Gram-positive and diptheroidlike (FIGURE 4*d*). Extremely rough variants are observed up to 14 days of incubation. The

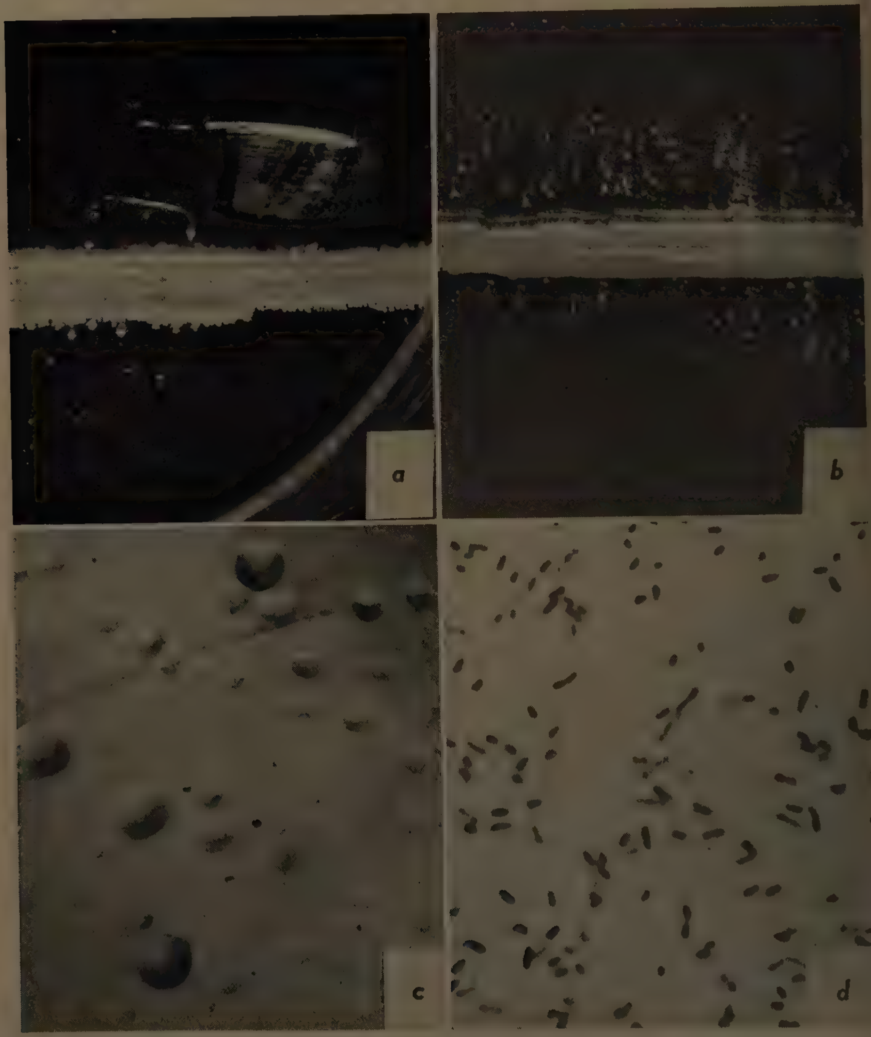


FIGURE 3. Reversion of PPLO strain V1 (Hull). (*a*) Colonies of *H. gallinarum* on the first plating of the first egg passage on blood-agar medium. The plate has been streaked with *Staphylococcus aureus*. A piece of agar was removed from this plate and transferred t another plate (shown in *b*). (*b*) Satellitism is demonstrated in this subculture. Reversio from the PPLO phase to the bacillary phase of strain V1 occurred at this stage. (*c* and *d* Reversion of PPLO strain J1 showing the dissociative phases of *H. gallinarum* on the surfac of solid medium. (*c*) Mixture of S- and R-type colonies on blood-agar medium, incubate 18 hours. (*d*) Gram-negative coccobacilli taken from the colonies in *c*. ×2000.

olonies enlarge and are completely flat, more granular, and more opaque (FIGURE 5*a* and *c*), while others produce daughter colonies on their surfaces (FIGURE 5*b*).

The Gram-variable organism that we recovered from the J and C strains was changed to *H. gallinarum* by passaging through the yolk sac. Single

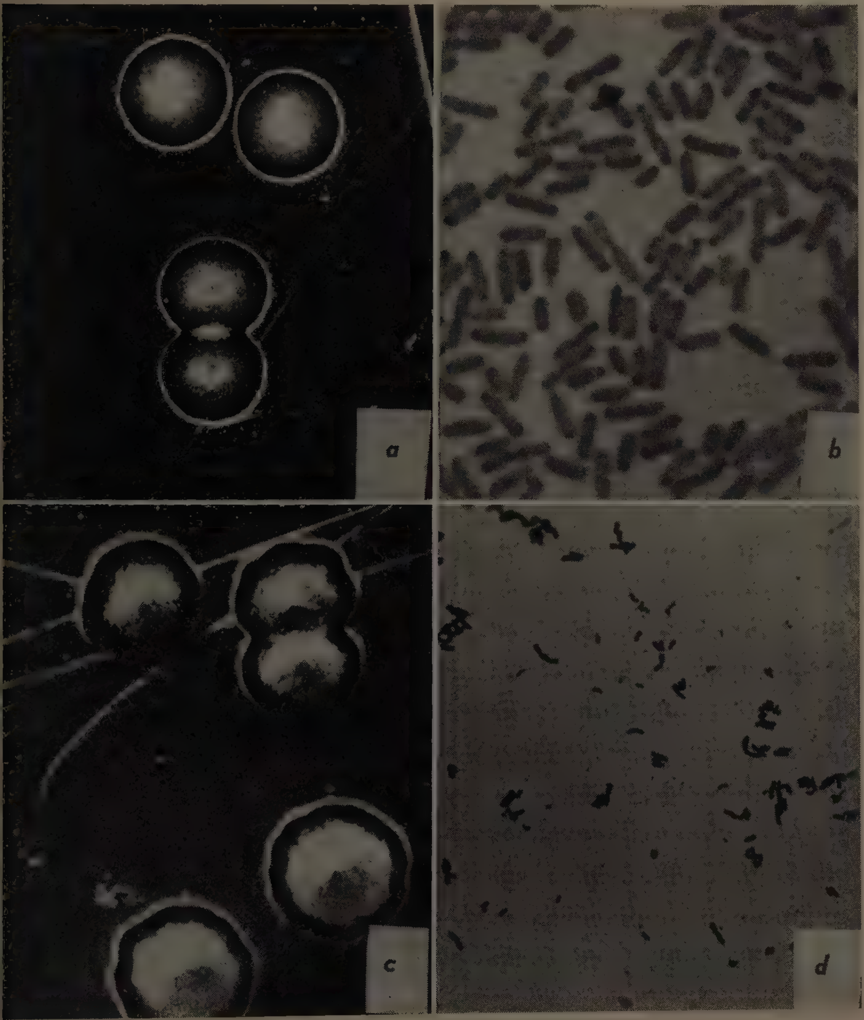


FIGURE 4. Reversion of PPLO strain J1 showing the dissociative phases of *H. gallinarum* n the surface of solid medium. (*a*) R-type colonies after 48 hours of incubation. They are enlarging and becoming slightly granular and opaque. (*b*) Gram-variable bacilli from the colonies in *a*. They are bipolar or appear septate and are longer and thicker. This type was recovered from strains J1, J4, and the C strains after these had been passaged in eggs and cultured on sheep-blood agar. ×2000. (*c*) R colonies aged at room temperature for 7 days. They have a central granular portion, a contoured surface, an intermediate flattened portion, and a beveled irregular periphery. (*d*) Bacilli stained from plate shown in *c*. They are Gram-positive and diphtheroid in morphology. ×1000.

colonies were subcultured in beef-heart infusion broth, and 0.1 ml. of a 24-hour growth was injected into the yolk sac of one chick embryo. After two passages the J strains produced the S-type colony of Gram-negative organisms showing satellitism. Five of the C strains required 3 passages to do this; C6 required 2 passages.

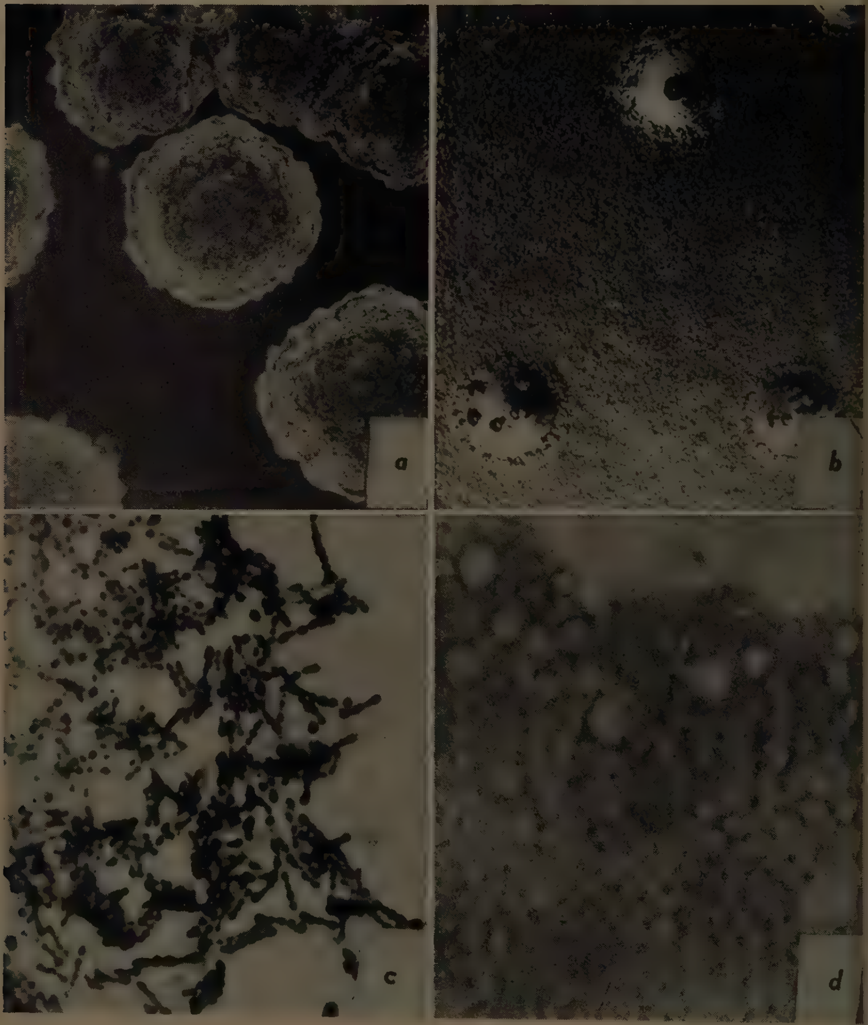


FIGURE 5. (*a*, *b*, and *c*) Reversion of PPLO strain J1 showing the dissociative phases of *H. gallinarum* in the surface of solid medium. (*a*) Extreme R-type colonies observed at two weeks' incubation time. They are larger, flattened, more granular, and more opaque. (*b*) Extreme R-type colony showing daughter colonies on the surface. (*c*) Taken from *a* and showing metachromatic granules, staining Gram-positive. (*d*) Reversion of PPLO strain J1. The edge of the colony on solid media after 4 days' incubation, showing L bodies.

Discussion

These experiments do not prove that L forms of bacteria and PPLO are the same, or that they are different forms arising from a single progenitor. However, the evidence should encourage further experimentation to prove or disprove such a relationship.

The importance of contamination of the egg source with *H. gallinarum* has been considered at all times. With strain 1499 the source of egg supply was changed, and the possibility of contamination by *H. gallinarum* was excluded by culturing earlier egg-passaged yolk material from passages Nos. 54 and 55. Batch controls on the eggs have been cultured in all experiments. We have never recovered *H. gallinarum* from uninoculated eggs in any instance.

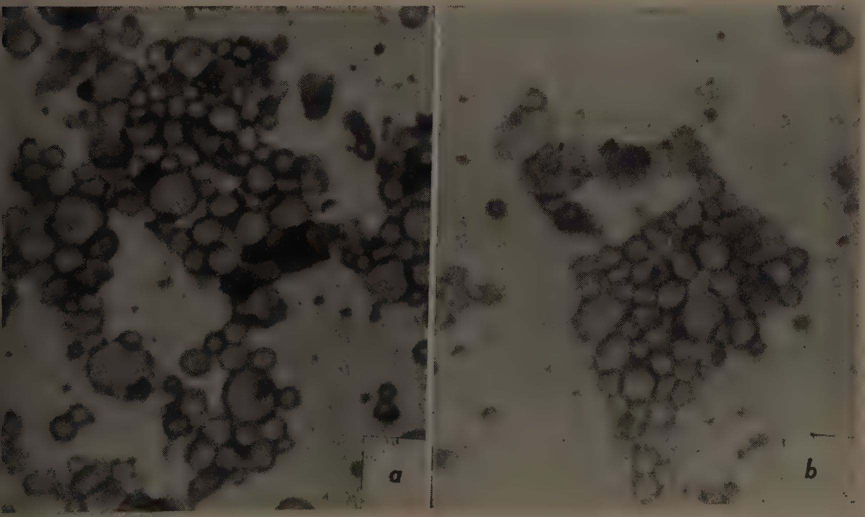


FIGURE 6. (*a* and (*b*) Colony shown in FIGURE 5*d* has been diluted on the surface of a tryptose-agar plate and mounted to show L bodies.

The spontaneously reverted strains were isolated only from egg-inoculated material that had been passed serially many times. In these experiments reversion did not occur in broth-passaged PPLO, one strain having been subcultured 32 times, while the J strains were transferred from 79 to 168 times. It appeared that some factor was present in the living tissue that favored reversion. A similar observation was reported by Wittler *et al.*,[5] who reverted a strain of PPLO during tissue culture. Strain 1499 reverted after 56 egg passages, and the bacillus remained stable in the S phase for 11 more passages. When we cultured frozen, stored material from egg passages Nos. 54 and 55, 5 passages were required before No. 54 reverted and 4 passages for No. 55. The avian PPLO or the S phase of *H. gallinarum* was not present when cultured on solid media during the intervening transfers, when examined by the dissecting microscope.

Reversion of the 4 strains by using heated saline extracts presents the problem of whether heat is an efficient method of destroying viable cell particles in the lysate of *H. gallinarum*. Under the conditions of the experiment and the number of transfers given to the control tubes, growth of the bacillus was not obtained. From other experiments we have found that heat at 60° C. for 30 min. in a rubber-stoppered immersed vial appears to kill the cells, which could not be recovered on culture.

In the reversion of the V strains using crude DNA extracts, controls on the extract in broth and in eggs were negative. Reversion did not occur in broth when the PPLO and extract were combined, but only when inoculated into the living embryo. The variation in the number of egg passages required to produce reversion—that is, V1, 1 passage; V2 and V32, 3 passages; and V36, 4 passages—prevents us from predicting when reversion would take place with any particular strain. The fact that many trials were unsuccessful indicates that there remain undetermined factors concerned with the specificity of the reaction. The dangers of depolymerization of the DNA during deproteinization also must be considered. Boivin,[25] in his studies with directed mutations in the colon bacilli, found that despite identical experimental conditions, the transformation of R_2 into R_1 was not produced regularly. Some tubes containing similar volumes of media, DNA, and bacteria transformed, while others did not.

Our observations on the dissociation of *H. gallinarum* are similar to those of Muriel H. Smith at the London School of Hygiene and Tropical Medicine, London, England, who isolated many variants of *H. influenzae* in the transition from S to R types. From our observations we conclude that *H. gallinarum* dissociates in a similar manner and that it produces many variants in the S to R transition, one of which is a stable form and resists reversion to the S type. Deacon *et al.*[9] studied *H. ducreyi* and isolated a Gram-positive bacillus which they called a smooth phase. Eliot and Lewis[26] reproduced infectious coryza in fowl with (according to the description) the S phase of *H. gallinarum*. In a few of their cultures they discovered a large, vacuolated, Gram-variable rod and a small Gram-negative rod. By repeated plating they obtained each in pure culture and produced coryza in the chick with the S type. They considered the Gram-positive rod as a contaminant. We suggest that it may have been an R variant of *H. gallinarum*. Hadley[8] in 1937 described organisms isolated from dwarf (d) colonies as being diphtheroid in form and Gram-positive, regardless of the reaction of the original strain to staining by Gram's method.

Dissociation of L forms and PPLO has not been studied intensively, and knowledge is lacking in this sphere. Shepard[27] has discovered the T colonies associated with PPLO. Yamamoto and Adler[17] differentiated avian PPLO into pathogenic and nonpathogenic types, the pathogenic type possessing coccoid forms and the nonpathogenic rings, filaments, and coccoid rods. These have been observed in L forms, and it appears that PPLO and L forms may dissociate in a similar manner.

Nelson[12] in 1936 made studies of infectious coryza in chickens. He divided this infection into three types based on clinical signs: a coryza of rapid onset and short duration produced solely by *H. gallinarum*; a coryza of slow onset and long duration produced by coccobacilliform bodies; and a coryza of rapid onset and long duration produced when both organisms were present. Is the second type a chronic manifestation of the first type, due to fixed L forms? The coccobacilliform bodies were later characterized and classified as *M. gallinarum* and are now considered to be the cause of CRD. In view of the doubt that exists concerning the origin of avian PPLO and the present difficulty of allocating these organisms to other bacteria, a precise definition of this species is difficult.

Binding proof to establish the relation of PPLO to bacteria will have to consist of the reversion of a classic strain of PPLO to a bacterium, reliably establishing the relationship of each, and finally producing the PPLO from the bacterium. It is essential that experiments be designed giving consideration to the bacterial genetics involved in microbial dissociation and the mechanisms of genetic exchange.

Summary

The results of experiments on the reversion of avian PPLO to *H. gallinarum* are presented. The reversion occurred only from organisms passaged in eggs and did not occur *in vitro*. Strains are described in which spontaneous reversion occurred, and strains in which reversion was induced experimentally by heated saline extracts of *H. gallinarum* and crude DNA extracts of the same organism. Some observations on the dissociation phases of *H. gallinarum* are recorded, and their implications with respect to the relationship of PPLO and L forms to bacteria are discussed.

Acknowledgments

The cooperation of E. L. Jungherr, Julius Fabricant, and A. S. Greig in supplying cultures used in this study is appreciated.

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DISCUSSION OF PART III

L. Dienes (*Massachusetts General Hospital, Boston, Mass.*): I agree with Edward and Klieneberger-Nobel that with sufficient experience one can distinguish from PPLO all the various L forms with which we are familiar. The PPLO constitute a well-defined group of independent microorganisms, but we do not know whether the L forms occur in nature or whether they may have importance in the biology of bacteria. However, the differentiation of PPLO from L forms, as I have explained in my paper, depends on quantitative differences in characteristics that are also variable in PPLO strains and that are of little value for the delineation of natural groups. I cannot regard the smaller size of PPLO as significant, because the prevalent size in both PPLO and L forms is variable and is influenced to a large extent by environmental factors. The reproductive processes, insofar as they are known, are similar in both groups, and the smallest organisms in PPLO do not represent specialized morphologic units different from the larger ones. The sterol requirement of parasitic PPLO may indicate the direction of investigation that will make possible a better characterization of these organisms. At present it cannot be used for the separation of PPLO from bacteria because some of the PPLO strains do not have this requirement. The characteristics by which we recognize PPLO and that unite these organisms in a group distinct from other organisms are their physical properties, small size, the structure of the agar colonies, and their lack of sensitivity to penicillin. These properties are the consequence of the absence of a rigid cell wall. Since many bacteria (such as L forms) can grow without a rigid cell wall, the properties mentioned define only a morphologic group that in other respects is heterogeneous. For this reason it seems to me premature to assign a place to the PPLO in the natural system. The difficulty is not unique to the PPLO: the classification of bacteria, even the definition of species is done, in many cases, on a questionable basis.

It is a different problem, and one important from the practical point of view, to define clearly which organisms should be included in the PPLO and which in the L forms, and to study carefully the cases in which the definition does not give a clear-cut answer. It is fortunate that these groups are among the best morphologic groups of bacteria. The definition that Sabin gave in 1941 and that I used later, with some modifications, is satisfactory at present. The PPLO group has lately acquired considerable importance. It has become necessary to determine the similarities and differences of strains, to arrange them into species, and to develop a usable nomenclature. This can be done without deciding upon some of the other basic problems of classification that confront us. To avoid complete chaos, the rules governing the selection of names are determined by international agreement. If, according to these rules, the only valid name for PPLO is *Mycoplasma*, we should accept it. The use of botanical names should not create a false impression of security in our present knowledge. The discovery of transduction and conjugation clearly illustrates how far we are from being able to make a true botanical classification of bacteria.

D. G. ff. Edward (*Wellcome Research Laboratories, Kent, England*): I have suggested that the PPLO represent a very neglected field of microbiology. I feel that one of the reasons for this is that undue attention has been given to only one aspect of their properties: I refer to the morphology. This has proved a very difficult problem to elucidate. The confusion that has existed has tended to deter, until lately, examination of the physiological, biochemical, and other properties of the organisms. In view of the uncertainty regarding their morphology, it has been difficult to classify them. Until recently I believed it premature to suggest a classification and nomenclature. I changed my opinion because I came to believe that detailed study of the group as a whole and of particular strains that were isolated would be encouraged by establishing a nomenclature and by defining species. It was for this reason that Freundt and I recently suggested a classification and nomenclature. Because the designations were regarded as provisional, they were made as simple as possible.

It is true that if the organisms are to be classified at all, an assumption must be made as to their relationship to other groups of microorganisms. I have stated my views on this previously and now can do no more than repeat them. There is nothing in this monograph to make me change my opinion. I regard the PPLO as quite distinct from any other microorganisms. They have a characteristic morphology and, as a result of the lack of cell walls, they are highly plastic. They produce characteristic colonies, and they have a peculiar metabolism, all except the saprophytic strains requiring sterols. Growth can be inhibited by antisera. These features distinguish them in general from bacteria or, at any rate, from bacteria in their normal phase. PPLO have certain similarities to viruses, such as the size of the smallest viable elements; their inhibition of growth by antisera also bears some resemblance to the neutralization of a virus. Nevertheless, their method of multiplication, their growth in artificial media, and their ability to grow extracellularly—even in tissue culture and in the tissues of animals—distinguish them from true viruses. The difficulty in deciding the relationship of the PPLO to bacteria resulted from the finding that bacteria can be transformed under certain artificial conditions into a different phase, the L phase, which bears a resemblance to PPLO.

I have said that the L phase *resembles* PPLO, and that is the crux of the argument. Is the L phase of a bacterium identical with the PPLO or is it not? Again, I can only repeat what I stated before, namely a belief that it is possible to distinguish between an L-phase organism and PPLO. There is no doubt that the two types of organism *resemble* each other. These resemblances are of great interest, but I think that the resemblances have been overemphasized and some of the differences neglected. I feel that the few L-phase organisms with which I have worked differ even in their microscopic appearances. I am no morphologist, but experienced morphologists describe differences in morphologic appearances and in mode of reproduction between the two classes of organism. I believe, from my own experience and from a reading of the literature, that it is possible to distinguish between colonies of L-phase organisms and the PPLO. Colonies of L-phase organisms still tend to retain some of their bacterial characteristics. This is especially obvious if the cultures are maintained for several days or if a semisolid medium is employed. Difficulties in distinguishing between two types of organisms have usually resulted from using opaque media and from relying on impression films for discrimination. The difference in nutritional requirements, shown by the need for sterols of all except the saprophytic strains of the PPLO, distinguishes them from the L-phase organisms, which do not require sterol.

In discussing these problems, consideration must be given to the recent claims that some of the PPLO have transformed into bacteria either in artificial culture or in tissue culture. In considering such claims, I think certain questions should be asked. First, are the organisms that have reverted undoubtedly true PPLO or were they the L phases of bacteria? Second, has it been clearly shown that the bacterium recovered was not a contaminant originally present in the culture of the PPLO? When it is claimed that one of the PPLO has reverted to a bacterium, both the PPLO and the bacterium should be shown to have an identical or very similar antigenic structure and similar biochemical behavior (at any rate, in regard to their ability to ferment carbohydrates), for these similarities have been demonstrated when bacteria have transformed into an L phase.

I feel that this question is very much open at the moment. However, I do not believe that these doubts should preclude us from classifying and naming species of the PPLO for, as I have already stated, I believe such a classification and nomenclature would be of enormous practical benefit. It would seem, at any rate from our experience with the organism of bovine pleuropneumonia, which goes back to the last century, that an organism can exist in nature as one of the PPLO, with the characteristics that have already been stated and with no tendency to change to a bacterial form. It is, of course, tempting to speculate on the evolutionary development of these organisms and to wonder whether they have been derived from bacteria in the course of time. However, it is equally possible to believe that the bacteria were developed from PPLO by evolution. Even if it is believed that these organisms are unstable under laboratory conditions (that is, that PPLO can change into bacteria), it is also equally possible that bacteria can change, through an L phase, to PPLO. Are we, therefore, to be inhibited from naming the PPLO because they may have been derived from bacteria? It might be claimed with equal justification that we cannot name bacteria because they may have been derived from the PPLO.

E. KLIENEBERGER-NOBEL: PPLO occur in nature in a large number of well-defined species as parasites and saprophytes. There are the PPLO of cattle and goats and the agalactia of sheep and goats, which have been known and extensively studied for decades; suspicion of their derivation from bacteria has never been voiced. Since 1930 several new species have been found in animals, in man, and in sewage and soil. Recently the idea of conversion of these organisms into bacteria, and vice versa, has appeared. The idea of conversion, however, is an old one. During my life, reports of the transformation of bacilli into sarcinae, of *Escherichia coli* into enterococci, and even of a banal mold into tubercle bacilli have appeared in the literature. These reports stirred interest and controversy, but finally all were consigned to oblivion. However, one should not underrate the difficulties of isolating PPLO and of keeping them alive and in pure condition on media that cannot be sterilized in the autoclave, as well as in embryonated eggs and tissue cultures.

In contrast to PPLO, the L form of bacteria is a laboratory product. The few cases in which unstable L forms were cultivated from pathological specimens do not prove that the organisms were present in this growth form in the infectious process because, on transfer to agar media, bacteria may grow out in the L form. The L form invariably reveals its bacterial nature when closely and critically studied. On suitable media the labile L form reverts to its mother bacillus when antibiotics are not present. The stable L-form growth is much coarser than the delicate PPLO growth. When the mother bacterium is known and both forms can be examined biochemically and serologically, a close relationship can be established.

A point of difference that I think essential is one that can be found only by electron microscopy and by properly designed filtration experiments. The PPLO produce a phase consisting of a great number of minimal reproductive units or granules, all of the same size (about 125 mμ in diameter), which the L form lacks. Both PPLO and the L form have elements varying in diameter from 250 or 300 to 500 or more mμ. These elements, of 300 mμ, are filterable through certain filters, but do not occur very frequently in the L form. The numerous very small elements of the PPLO (125 mμ) can be compared in size to the elementary bodies of larger viruses. They are very condensed.

The L form, it seems now, is a bacterium without a rigid cell wall. The PPLO constitute a family or order of their own. Such a far-reaching conclusion as that involving transformation of PPLO into L form and bacteria, and vice versa, should not be drawn unless the purity of the original culture has been thoroughly established and the transformation convincingly demonstrated.

E. A. Freundt (*Statens Seruminstitut, Copenhagen, Denmark*): The personal interest I have shown for several years in the morphology and mode of reproduction of the PPLO may have given the impression that I attached excessive importance to these properties in the classification of the organisms. As mentioned in my paper in this monograph, a precise and correct morphologic description of the PPLO is undoubtedly fundamental to our understanding of their taxonomic relationships since, in the words of Dienes, "The natural system of organisms is based mainly on structure and mode of reproduction." On the other hand, I quite agree that other important properties, such as cultural, biochemical, and serologic characteristics, should of course be considered on an equal footing with morphology in the discussion of the possible relationship of PPLO to bacteria in general and to bacterial L-phase variants in particular.

Morphology

The assumption of a close relationship between the PPLO and the L forms was originally based on the observation of a striking similarity in their colonies. Although it is usually possible to distinguish between them, I am inclined to agree with Dienes that the features that enable us to differentiate may in themselves be of questionable importance from a taxonomic point of view. The decisive point is whether a true morphologic similarity exists on the cellular level.

I need not repeat here my reasons for maintaining the view that the morphology and mode of reproduction are fundamentally different in PPLO and L-phase variants. I emphasize once more that the development of branching mycelia is, in my experience, a distinct and regularly occurring property of the PPLO, which cannot be compared with the tendency of some bacteria to form filamentous, occasionally branching cells during growth, such as occurs on deficient media or under the influence of penicillin. Neither can the nonuniform and scattered granules that may occur in bacterial filaments be compared with the uniform, regularly spaced, coccoid elements that develop in the PPLO filaments. I know from experience that it is difficult to substantiate the claim of a regular occurrence of mycelial elements in all PPLO strains. Frequently the mycelia are so small that they can hardly be discerned with the light microscope and are very liable to be distorted or even destroyed by the procedures necessary for electron-microscope studies. In successful electron micrographs, however, mycelial elements are often present in rather large numbers, no matter which strain is examined.

The fact that the physical properties of the cell envelope apparently are similar in PPLO and L-phase variants need not imply similarity as to anatomical and chemical structure. A similar soft and fragile cell envelope is found, for example, in the myxobacteria. Actually, the demonstration in the PPLO of repeatedly branching filaments that are able to maintain their distinct external shape in a liquid medium provides in itself sufficient evidence that their cell envelopes must differ in structural properties from those of the protoplastlike L forms.

Growth Requirements and Metabolism

The similarity between PPLO and L-phase variants, with respect to growth requirements, is limited to their common, although not invariable, dependence on components such as ascitic fluid or serum. However, on this point, also, the agreement is probably only apparent since, in the two cases, the active factors are of a different nature and mode of action. The vast majority of PPLO depends on cholesterol as a nutritional factor. The fact that cholesterol is not required by *Mycoplasma laidlawii* does not minimize the value of cholesterol dependence as an important distinguishing mark between PPLO and L forms, so long as this or related substances have not been shown to be necessary for any L organism of known bacterial origin. The exact requirements of the L-phase variants are not known, but studies by Medill and O'Kane (1954) suggest that the growth-promoting property of serum on the L-phase variants depends, at least in part, on a neutralization of growth-inhibiting components in the basic medium. Experiments with synthetic media (Smith, 1955; Medill and O'Kane, 1954) also suggest the requirement of much more complex media for the PPLO than for the L-phase variants.

Edward's phenomenon, the production of the peculiar precipitates known as "film and spots," signifies another metabolic property distinguishing several PPLO strains while, to my knowledge, this has not been demonstrated with any L-phase variants.

The fermentation pattern of the PPLO able to attack sugars is remarkably uniform in contrast to that of the L-phase variants, the fermentative properties of which depend entirely on the activities shown by their normal bacterial ancestors.

An interesting difference between PPLO and L forms is suggested by the observation of Kandler *et al.* (1956) that PPLO were unaffected by various respiratory poisons, while the L phase of *Proteus* was as sensitive as the normal bacterium. This observation is in agreement with experiments reported by Rodwell and Rodwell (1954), although Lecce and Morton (1954) were able to abolish the oxygen uptake of human PPLO strains by cyanide poisoning.

Reaction to Antibiotics

The similarity of PPLO and L forms in their reaction to antibiotics, in particular their common high resistance to penicillin, cannot be ignored, of course. However, our present lack of information as to the mechanism of penicillin resistance in PPLO and L forms does not allow too far-reaching conclusions in this field. The similarity of their reaction to penicillin may be based on similarities of the structure of the cell envelope; further studies are awaited with interest.

Pathogenicity

The pathogenicity patterns of PPLO and L forms are quite dissimilar. The PPLO include a variety of highly pathogenic to completely nonpathogenic species, in addition to species of doubtful pathogenicity. Some of the pathogenic PPLO present remarkable common properties, such as the particular affinity for the interstitial connective tissues, for the serous membranes, and for the joints, as seen for example with both varieties of *Mycoplasma mycoides* and with *M. agalactiae*, *M. arthritidis*, and *M. hyorhinis*. In contrast, the L-phase variants are generally, insofar as is known, nonpathogenic—notwithstanding the possible pathogenicity of their bacterial mother strains. This was clearly demonstrated by Silberstein (1953) with stable variants of *Salmonella typhimurium*, and by me in studies with the L forms of *S. moniliformis* and of highly virulent Group A *Streptococcus*. Similar observations were reported briefly by Dienes and his collaborators, and by Minck (1950) and Tulasne (1951). The retained ability to produce endo- and exotoxins as shown for the L forms of *Vibrio cholerae* and *Clostridium tetani*, respectively (Tulasne and Lavillaureix, 1954, 1955, and Scheibel and Assandri, 1958, personal communication), is not inconsistent with the general experience that loss of infectivity is characteristic of the L-phase variants.

Conversion of PPLO to Bacteria?

I am in complete agreement with Dienes that the present evidence of reversion of recognized PPLO to bacteria is apparently inconclusive. The observations of supposed conversion to corynebacteria previously reported by Wittler *et al.* (1956) and by Smith *et al.* (1957) deserve particular consideration. It should be added that the "Campo L" strain studied by the latter group of workers is generally recognized as a true PPLO, since it was classified by Edward as *M. hominis*, Type 2 (Nicol and Edward, 1953).

Wittler found that the biochemical properties of her *Corynebacterium* differed in at least two respects from those of the PPLO strain, from which it was supposed to arise. This observation in itself furnishes strong evidence against the belief that the PPLO in question could be the L phase of the *Corynebacterium*, since it is a general experience that the biochemical properties of the

L phase are always identical with those of the normal bacterium. Neither Wittler *et al.* nor Smith *et al.* were able to reconvert the corynebacteria to L-type or PPLO colonies. The comparative serologic investigations, which suggested some antigenic relationship between the PPLO and the corynebacteria, should be continued. Altogether, it may be said that the available data are apparently not sufficient to exclude the possibility that the corynebacteria, which were isolated from the PPLO cultures, were merely accidental contaminants. Should it be possible, nevertheless, to prove decisively that a reversion has taken place, this could only be interpreted to indicate that the strains in question were erroneously classified as PPLO originally. Generalization from such observations to the effect that PPLO as a whole may be characterized as L-phase variants is not permissible a priori.

In conclusion, the similarities found between the PPLO and the L forms are, with a few possible exceptions, merely superficial and inconclusive. Actually, it is possible to point out profound differences between the two groups of organisms.

The L-phase variants are associated only because they represent one biological phenomenon while, taxonomically, they constitute a highly heterogeneous group.

The general properties of the PPLO, including their morphology and mode of reproduction, as well as their cultural, biochemical, and pathogenic properties, in my opinion provide convincing evidence that they constitute an autonomous taxonomic group and should be classified accordingly. Their distinctiveness in several respects, also, when compared with bacteria in general, inspired the proposal by Edward and myself to establish a separate order, the Mollicutales or Mycoplasmatales, for the PPLO to be placed within the Schizomycetes.

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KURT LIEBERMEISTER (*Bacteriologisches Institut am Krankenhaus rechts d. Isar der Stadt München, Munich, Germany*): It is not within the framework of this conference to decide whether the PPLO and L forms of bacteria are phylogenetically related. Thus far we have no proof that the PPLO evolved from the L forms. However, this question is sufficiently interesting and important to be studied further.

As groundwork for all further considerations and conclusions it is indispensable to study, and, in accordance with our present knowledge, to define the following points:

(1) Properties common to the PPLO and stable L forms (3A colonies) with regard to morphology, cytology, multiplication, morphology of cultures, metabolism, conditions of growth, and physical and serologic properties.

(2) Practically applicable objective possibilities of differentiation.

Concerning the classification and nomenclature of the PPLO, it will be necessary to devise a special microorganism group. Considering their morphology, it appears advisable to use the designations spherical, cystic and, possibly, pleomorphic in the nomenclature, but no term should be used for PPLO that suggests filamentous structure.

I suggest the following nomenclature for the L forms: (1) incomplete spheroblasts of *Proteus* (or such as *coli*), 3B colonies (after Dienes), and (2) complete spheroblasts of *Proteus* (or such as *coli*), 3A colonies (after Dienes).

Although nomenclature is a necessary evil, its problems seem to be almost more the task of the philologist than the experimenter.

JEAN BRISOU (*School of Medicine of the University of Poitiers, Poitiers, France*): The agent for bovine pleuropneumonia has been known since 1898, when it was discovered by Nocard and Roux.[1] It does not seem inappropriate to mention here that, from the very beginning, French investigators made an intensive study of this microorganism. In fact, Borrel and Salimbeni[2] produced its first cultures by the technique of collodion bags introduced into the peritoneum of the guinea pig. Dujardin-Beaumetz[3] and Borrel, Dujardin-Beaumetz, Jeantet, and Jouan,[4] followed by Bordet, successfully grew cultures *in vitro* in serum bouillon or on blood agar. Minute descriptions of the agent responsible for pleuropneumonia were given in the course of these investigations; the picture is classic.

Borrel and his collaborators chose the term *Asterococcus mycoides* to designate the new pathogenic agent. Since then, numbers of similar microbes have been found in a variety of animal and human diseases.

As specimens multiplied, classification became necessary. Successively suggested were the terms Borrelomycetales (Turner, 1935),[5] Mollicutales (Edward, 1955),[6] and Pleuropneumoniales (Tulasne and Brisou, 1955).[7] Freundt, in 1955,[8] preferred to regroup the microorganisms in the order Mycoplasmatales, with a single genus, *Mycoplasma*, which he borrowed from Nowak (1929).[9]

Whatever the designations chosen, the bacteria were defined thus: infinitely small, very pleomorphic entities, reproducing themselves by the fragmentation of filaments into elementary, coccoid, filterable bodies. These elements are extremely fragile; in fact, rough handling destroys them. The organisms produce no spores, are immobile, and do not take Gram stain. Cultures are easily obtained on enriched media; on agar the colonies are observed to be microscopically small. Finally, these bacteria never are transformed into bacteria of normal size. Pathogenic types and commensals are known. These are all classic, well-established concepts that do not require further elaboration.

For these very distinctive microbes that are filterable but culturable *in vitro* without the aid of living cells, Tulasne and I, in 1955, proposed to create the order Pleuropneumoniales.[7] This term has the advantage of embodying a reminder that the first representative of the order is responsible for bovine pleuropneumonia. Historically, of course, it would have been preferable to use *Peri*pneumoniales, but the clinical and histopathological behavior of the disease further justified the appellation of our choice; it was quickly adopted by the French-speaking countries, and it seems pointless to change it.

Only one family, which we named the Pleuropneumoniaceae, would be considered as belonging to Pleuropneumoniales and one genus, *Pleuropneumonia*, the type species of which is represented by *Pleuropneumonia bovis*.

We have been reminded, very properly, that it would be desirable to return to the epithet chosen by Borrel and his collaborators.[4] Nothing could be simpler, for the term *Asterococcus* has been in official use since 1910, and it is natural and fair to accede to its incontestable priority. In that case we can keep the very convenient order Pleuropneumoniales, with a family Asterococcaceae and at least one genus, *Asterococcus*, with a type species *Asterococcus mycoides*. Thus, all the germs included in the genuses *Pleuropneumonia* or *Mycoplasma* will become *Asterococcus*. One cannot help wondering why more than ten different names have been proposed since 1910 to designate a perfectly well-known and officially labeled genus and species!

In concluding this introduction it is proposed, therefore, to keep the order Pleuropneumoniales (Tulasne and Brisou, 1955)[7] and to consider this order as having a single family, the Asterococcaceae, with a first genus, *Asterococcus*. I shall show that perhaps there is room for other families—certainly, for other genuses and species.

The L Forms and PPLO

As early as 1923, during the time when the filterable forms of bacteria were being discovered, the work of Fontes,[10] of d'Herelle,[11] of Hauduroy,[12] and of de Jong[13] had established the existence of filterable forms of many bacteria, notably *Shigella*, *Salmonella*, and the coliforms. These inframicrobes were found to be capable of passing through filters, not only in cultures subject to the action of bacteriophages, but *in vivo* in the blood or salts of the ill or convalescent. The extraordinary resistance of these filterable microorganisms was made known through the researches of d'Herelle and Hauduroy,[11,12] who estimated one of them, *Shigella dysenteriae*, to be capable of enduring for at least ten years.

Since these investigations, the literature has been much enriched by interesting observations that lead me to believe that all microbes pass through a filterable phase in the course of certain reproductive cycles. It would be out of place in this paper to discuss the course of investigation from which these concepts emerged: questions concerning the normal cycle of reproduction, waiting, dormant, resistant phases, and results of phagic action or of antibiotic action are discussed elsewhere. To stay within the limits of taxonomy, let us emphasize that the filterable forms, the L forms of Klieneberger-Nobel,[14] suggest certain aspects of the Pleuropneumoniales. Under certain conditions, the microbes seem to take on the appearance of the pleuropneumonia agents, and this may cause confusion.

In the period of which I have been speaking, the PPLO also were discovered; they, too, are filterable, resemble the Pleuropneumoniales, are culturable, and are very widely distributed throughout nature. Thus, the expression "pleuropneumonialike organisms," which seemed so apt in the beginning, has now become a source of error. From the moment when it was suggested that the filterable forms of microbes and the L forms resembled the PPLO, every effort was made to establish increasingly close relationships between microorganisms that have, without doubt, nothing in common.

The microbe world long ago accustomed us to pleomorphism, always one of its characteristics. It should not astonish us that unicellular beings, so tiny but bearing extraordinary enzymatic potentialities, for reasons of self-perpetuation should be obliged to adapt themselves to the variations in environment to which they are exposed ceaselessly. The bacteria respond to given life conditions by different methods of reproduction or by dormant periods that are phases of resistance. That in the course of these reproductive cycles the microbes temporarily resemble the Pleuropneumoniales is not so surprising.

As Tulasne and I proposed in 1955,[7] let us separate the two problems. On the one hand, there are the filterable forms of bacteria, to which we gave the name "Bactepneumoniales,"* a simple mnemonic term with no taxonomic pretensions, serving only as a reminder that under certain circumstances the bacteria can show a resemblance to the PPLO. On the other hand, there is, in the order Pleuropneumoniales, the genus *Asterococcus* as already defined.

An essential characteristic of the L forms is reversion to the normal bacterial form. Everyone agrees on this point. This is not a case of fixed types, as all the experimentation carried out since the work of d'Herelle and Hauduroy amply demonstrates. Thus, the order Pleuropneumoniales is closed to these transitory forms.

In the case of the PPLO, it seems essential to emerge from the ambiguity in which we have floundered for the past several years. It is accepted that there exist filterable, culturable microorganisms that resemble the Pleuropneumoniales closely enough to be mistaken for them. These bacteria are found in nature and in the natural cavities of man and animals. If these microorganisms sufficiently resemble Pleuropneumoniales to be mistaken for them, why not accept them as belonging to that order? Are they or are they not Pleuropneumoniales? That is the dilemma. If the decision is affirmative, let us form a genus for them in the order Pleuropneumoniales; if negative, let us place them elsewhere but abandon the PPLO classification, which is not at all taxonomic. From a single fact—possible resemblance between microorganisms—a concept has been formed and a term coined! However, it does not seem too difficult to escape from this confusion. Hauduroy[12] recently proposed the epithet *Para-asterococcus*. In case this appellation fails to win votes—and, in fact, the prefix *para* is never desirable in nomenclature—a more precise term must be found.

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* This term seemed more explicit than "L form," but perhaps diabacteria (such as *Diagoholoym* or *Diaproteus*) or ataxobacteria would be preferable.

Since these microorganisms resemble and seem to imitate *Asterococcus*, I thought that the expression *Mimasterococcus*, from the Greek *mimesis* (imitation), would be suitable. Thus, the order Pleuropneumoniales would include all those microbes that are filterable, culturable, and nonreversible to the normal bacterial forms, as distinguished from the rickettsiae, the Chlamydozoa, and the viruses that grow only on living tissues.

In Pleuropneumoniales, two genuses belonging to the same family, the Asterococcaceae, may be visualized. These genuses would be *Asterococcus*, for animal or human pathogenic forms, and *Mimasterococcus*, for saprophytic or commensal specimens. Most of these microbes will correspond to the present PPLO.

It would then be accepted that the L forms, which are filterable, dwarfed, and reversible from bacteria, represent a separate problem. Transitory, deceptive morphologies present diagnostic difficulties that are not always easy to solve. The morphologic similarities are indisputable, but the bacteriologist now has at his command technical methods that were perfected in recent years by those who patiently have prospected the world of the infinitely small.

Conclusions

A possible and practical taxonomy for the group of microorganisms under discussion may be summarized in this way:

Order, Pleuropneumoniales
(transition between eubacteria and rickettsiae)

Family, Asterococcaceae

Genus I, *Asterococcus*
Type species, *Ast. mycoides*
(including only pathogenic species)

Genus II, *Mimasterococcus*
Type species, *Ast. laidlawii*
(including all commensal and saprophytic species)

The term PPLO is not taxonomic, but only a more or less practical symbol for microorganisms that have transitory morphologic similarities. The filterable forms of microorganisms and the L phases of their cycles are also not taxonomic groups.

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D. G. ff. Edward: I consider that in the discussion on the classification of the pleuropneumonia group of organisms a greater degree of agreement was reached than might appear at first sight. I have, therefore, consulted privately E. Klieneberger-Nobel, L. Dienes, E. A. Freundt, and M. Rubio-Huertos, who were members of the panel. Unfortunately, owing to language difficulties, I have not spoken to J. Brisou. I am able, therefore, to make the following statement, which I hope will be of assistance to those working in this field. This is an agreed statement and represents the views of those mentioned.

We consider that there is a group of organisms occurring in nature with clearly defined properties; these are known as organisms of the pleuropneumonia group (PPLO). The organisms belonging to this group can be classified and named. It is generally agreed that the type species is the organism of contagious bovine pleuropneumonia. If the international laws of nomenclature are to be observed, there is only one valid name for this organism, namely, *Mycoplasma mycoides*. The rules of nomenclature cannot be altered and it is impossible to discard this name merely because one does not like it.

A provisional classification and nomenclature that conforms to the international laws of nomenclature has been suggested by E. A. Freundt and myself; in this, specific names have been given to all the recognized species. This nomenclature must be regarded as provisional and, for that reason, one genus only has been suggested. At a later stage when more is known it may be considered advantageous to establish other genera.

There is another group of organisms that we consider to be distinct from *Mycoplasma*, although they resemble PPLO in their morphology and colonial appearances. These organisms are derived from bacteria under artificial conditions and also, possibly, in nature. Where an organism is so derived from a bacterium and can be subcultured in series, whether or not it will revert to the bacterium in the absence of the conditions that originally induced the pleuropneumonialike form, it will be known as the L-phase variant of the bacterium. The term L form, which has caused so much confusion, should be restricted to morphologic changes only.

There has been some disagreement as to whether *Mycoplasma* can change into bacteria. Our opinion is that this has not been proved. Reasons have been given for believing that it is unlikely. For example, it has been pointed out that, in most instances, if an organism turns into an L phase it loses its pathogenicity. It is well known that many *Mycoplasma* species are pathogenic. It is, therefore, our opinion that the onus is on those who claim that a *Mycoplasma* has changed to a bacterium to show that both the *Mycoplasma* and the bacterium possess the same genetically inherited characters.

If an organism is considered to be a true *Mycoplasma*, it is necessary to identify its species by examining its properties and comparing them with those described for the species. To facilitate this it is necessary that representative strains of the type species should be maintained in a collection of type cultures.

Part IV. Physiology

CHEMICAL COMPOSITION OF PPLO*

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The ability of the so-called pleuropneumonialike organisms (PPLO) to pass through bacteria-retaining filters, as well as their pleomorphism and poor staining quality, have helped to distinguish these microorganisms from bacteria.[1-3] The introduction of stable L forms of bacteria[4-6] and the interest in bacterial protoplasts stress the need for more detailed information concerning this group of microorganisms. Therefore a chemical analysis was carried out using strain O7, isolated from a human urethra. Later, for comparison purposes, another human strain, Campo L, an avian strain J, a bovine strain B-15, and a saprophytic strain Laidlaw B, were examined to a limited extent. Strain O7 was chosen because of the relatively good yield of cells that can be obtained in a heart infusion medium[7] supplemented with 1 per cent "Bacto-PPLO" serum fraction.[8] Culture techniques have been described previously.[9] Cells were harvested from 10- to 20-liter quantities of broth by centrifuging them in a Sharples super centrifuge. The harvested cells were washed several times with physiological saline, resedimented each time in a Servall angle centrifuge at 12,000 rpm. The cellular nitrogen was estimated turbidimetrically[10] as well as by a micro-Kjeldahl method.[11] The methods employed for the estimation of the nucleic acid content and composition have been described previously.[9] The lipids were extracted with methanol and ether, evaporated to dryness under vacuum. They were chromatographed on a silicic acid column and eluted in a manner similar to that described by Marinetti *et al.*[12] Cholesterol was determined by the Liebermann-Burchard reaction. Fatty acids were titrated with potassium hydroxide.[13] Cholesterol and cholesterol esters were estimated by separation on a silicic acid column[14] and by paper chromatography based on the method of Neher and Wettstein.[15]

A summary of the data obtained to date is presented in TABLE 1. Each of these values is based on several different determinations. From such data it can be seen that the protein makes up approximately 40 per cent of the dry weight of the organism. Another 4 per cent may be attributed to methanol:ether extractable lipid. An interesting facet of this lipid fraction is the fact that it contains a significant amount of sterol. This will be discussed more fully later in this paper. Also, it may be noted from TABLE 1 that nucleic acids are estimated to comprise nearly 6 per cent of the dry weight of this organism. Further inspection of this nucleic acid fraction shows that the

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pentose-containing type is present in an amount twice that of the desoxypentose-containing type. A further summary of the nucleic acid composition presented in a previous publication[9] is shown in TABLE 2. It may be noted that the amount of adenine and thymine is slightly larger than that of guanine and cytosine. In the instance of pentose nucleic acid the bases guanine and cytosine are present in larger amounts than adenine and uracil.

TABLE 1
CHEMICAL ANALYSIS OF PPLO 07

Fraction	Mg./100 mg. dry weight	
	Mean	Range
Protein	41.2	31–42
Nucleic acid	5.8	4–6
Pentose	3.9	3–5
Desoxypentose	1.9	1–3
Lipid	4.0	3–5
Fatty acid	0.23	0.2–0.4
Sterol	0.68	0.7–0.9
Phospholipid	3.06	2.2–4.7

Ratios

Phospholipid phosphorus (residue/supernatant)	1.5
Phospholipid phosphorus/cellular nitrogen	0.02
Total sterol/cellular nitrogen	0.12

TABLE 2
MOLAR PROPORTIONS OF BASES OF NUCLEIC ACIDS OF PPLO STRAIN 07

Base	Desoxypentose	Pentose
Adenine	10	10
Guanine	8.5	17.5
Cytosine	10.4	16.8
Thymine	11.4	—
Uracil	—	11.2
Purine/pyrimidine	0.86	0.98
$\frac{\text{Adenine} + \text{thymine}}{\text{Guanine} + \text{cytosine}}$	1.13	—

TABLE 3 shows a further analysis of the lipid fraction of this microorganism. The residue and supernate fractions were obtained by exposing washed whole cells of the organism to vibrations in a 9 kc. Raytheon magnetostrictor vibrator for 30 min. The resulting lysed cells were subjected to centrifugation for 30 min. in a Servall angle centrifuge at 12,000 rpm. The resulting supernatant was clear. The sterol was precipitable as a digitonide and gave a positive Liebermann-Burchard reaction. The free sterol is digitonin-precipitable without prior hydrolysis. The sterol was shown to be identical with cholesterol by its R_f value and color reaction on paper chromatography. From these data it appears that the amount of free sterol in the supernatant fraction is only slightly higher than that present in the residue fraction. A significantly larger amount of bound sterol is present in such residues. It can be noted further that the bound sterol is more than twice as prominent as the free sterol in this organism. In the phosphorus analyses, the supernatant fraction contains more of the acid-soluble phosphorus, whereas the level of phospholipid is higher in the residue fraction. Very little phosphoprotein (hydrolyzable in hot 18 *N* H_2SO_4) is detectable in either fraction. Further data on the sterol content and distribution among certain strains of PPLO are presented in TABLES 4 and 5. From this data it may be seen that the saprophytic strain Laidlaw B and the bovine strain B-15 do not contain a detectable amount of sterol. Also, the avian strain J contains significantly more sterol, in the form of bound sterol, than the human strains examined. TABLES 6 and 7 contain data pertaining to

TABLE 3
ANALYSIS OF LIPID FRACTION OF PPLO STRAIN O7

Cell fraction	Sterol (mg./gm. dry weight)		
	Free	Bound	Total
Whole	2.04	5.59	7.63
Residue	0.95	3.30	4.25
Supernatant	1.09	2.29	3.38
	Phosphorus-containing components (mg./gm. dry weight)		
	Phospholipid	Acid-soluble phosphorus	Phosphoprotein
Whole	1.38	12.80	0.05
Residue	0.82	5.05	0.05
Supernatant	0.56	7.75	0

TABLE 4
STEROL CONTENT AND DISTRIBUTION AMONG VARIOUS STRAINS OF PPLO

Sterol	Strain				
	O7	Campo	J	B-15	Laidlaw B
Total sterol (mg./mg. cellular N)					
Whole cells	0.12	0.16	0.22	0	0
Residue	0.06	0.10	0.10	—	—
Supernatant (by difference)	0.06	0.06	0.12	—	—
Free sterol (mg./mg. cellular N)					
Whole cells	0.03	0.04	0.03	0	0
Residue	0.01	0.02	0.03	—	—
Supernatant (by difference)	0.02	0.02	0.01	—	—
Bound sterol (mg./mg. cellular N)					
Whole cells	0.09	0.12	0.19	0	0
Residue	0.05	0.08	0.10	—	—
Supernatant (by difference)	0.04	0.04	0.09	—	—

the content and distribution of the sterol in the human strain O7 at various stages of growth. Estimates on whole cells show that the total and free sterol increase to a maximum at the end of 24 hours' growth and then drop by 32 hours, only to rise again to the same maximum level at 48 hours. With the bound sterol this is less prominent. This same phenomenon is reflected in the supernatant fraction of this organism. In the residue portion the total bound and free sterol increases in amount gradually over the entire incubation period. The ratio of bound:free sterol remains essentially constant for all the fractions.

TABLE 5
DISTRIBUTION OF STEROL AMONG CERTAIN STRAINS OF PPLO

Sterol	Strain		
	O7	Campo	J
Bound/free			
Whole	3.0	3.0	6.5
Residue	5	4.0	3.3
Supernatant	2	2	9.0
Total sterol: residue/supernatant	1.0	1.5	0.8
Bound sterol: residue/supernatant	1.4	2.0	1.1
Free sterol: residue/supernatant	0.5	1.0	3.0

TABLE 6
STEROL CONTENT DURING THE GROWTH CYCLE OF PPLO STRAIN O7

Age (hours)	Sterol (mg./mg. cellular N)								
	Whole cells			Residue			Supernatant (by difference)		
	Total	Bound	Free	Total	Bound	Free	Total	Bound	Free
8	.07	.03	.04	.05	.04	.01	.02	0	.02
24	.22	.08	.14	.07	.04	.03	.15	.04	.11
32	.16	.07	.09	.13	.08	.05	.04	0	.04
48	.21	.09	.12	.14	.08	.06	.07	.02	.05
120	.22	.08	.14	.16	.10	.06	.06	0	.06

TABLE 7
DISTRIBUTION OF STEROL DURING GROWTH CYCLE OF PPLO STRAIN O7

Age (hours)	Bound/free			Residue/supernatant		
	Whole	Residue	Supernatant	Total sterol	Free sterol	Bound sterol
8	0.9	5.6	0	1.8	0.2	∞
24	0.5	1.6	0.3	0.5	0.3	1.2
32	0.8	1.8	0	3.0	0.8	∞
48	0.8	1.2	0.5	2.2	1.3	3.5
120	0.6	1.4	0	2.7	1.0	∞

A difference in the proportion of volatile to nonvolatile acids between a human strain and an avian strain is shown in TABLE 8. A paper chromatographic analysis of the volatile fatty acids of the human strain O7 indicated that butyrate was the most prevalent. It was present in approximately four times greater amount than the next most prevalent, acetate. Only a trace of propionate was detectable. With the volatile fatty acid of strain J, acetate was the most prevalent, with some propionate detectable. No other fatty acids were noted.

The effect of endogenous respiration of whole cells of strains J and O7 on bound and free sterol in these organisms was examined by incubating washed whole cells in a Warburg respirometer for a period of 4.5 hours at 37° C. The data from such an experiment are shown in TABLE 9. With the human strain O7 there was an increase of free sterol during endogenous respiration with a concurrent reduction of bound sterol. This change was not as noticeable in the experiment using the avian strain J.

TABLE 8

FATTY ACIDS OF STEROL ESTERS FOUND IN CERTAIN PPLO

Strain	Fatty acid (μM)		Volatile/nonvolatile
	Volatile	Nonvolatile	
J	174	203	0.86
O7	228	5	49.5

TABLE 9

EFFECT OF ENDOGENOUS RESPIRATION ON BOUND AND FREE STEROL

Strain	Sterol				Ratio		
	Free		Total		Bound sterol/free sterol		Free before/Free after
	Before	After	Before	After	Before	After	
J	0.129*	0.183	0.142†	0.125†	—	—	.70
O7	0.136	0.241	0.375	0.335	1.8	0.4	.56

* Milligram per milligram cellular nitrogen.

† These estimates of total sterol are invalid as a red precipitate formed with the color reagent.

Summary

The chemical composition of several pleuropneumonialike organisms has been examined. An analysis of the nucleic acids of a human strain of this group of microorganisms indicates that in this respect they do not differ significantly from bacteria except possibly as regards their low quantity of total nucleic acid.

The lipid content, approximately 5 per cent of the dry weight of the cells, is similar to that of bacteria also. However, a distinguishing feature of the lipid fraction of PPLO isolated from human and avian strains is the presence of a sterol similar to cholesterol and cholesterol ester. These sterol compounds were not detectable in a saprophytic and a bovine strain of PPLO examined. The only other instance of the presence of a sterol fraction in bacteria[16] was probably not cholesterol and was present at a much lower concentration than that estimated in these organisms.

Also, there appears to be a significant difference between the fatty acid components of the lipid fractions of the avian and human strains examined.

Studies with the avian and human strains of PPLO examined for sterols after a prolonged endogenous respiration period show that the bound sterol is split, leaving the free sterol. The free sterol does not appear to be broken down by these organisms.

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NUTRITION AND METABOLISM OF *MYCOPLASMA MYCOIDES* VAR. *MYCOIDES*

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METABOLISM

My work on the carbohydrate metabolism of *Mycoplasma mycoides* has already been published.[1-3] FIGURE 1 shows the known catabolic reactions of this species and the probable pathways followed. Only one strain was examined, the V5 strain, isolated from a case of bovine pleuropneumonia in 1936. The cells were harvested from aerated cultures in a complex medium. The sole products of hexose oxidation were acetate and CO_2. It was concluded that hexoses were broken down through the glycolysis pathway, followed by oxidation of pyruvate by an α-lipoic acid, coenzyme A (CoA), diphosphopyridine nucleotide (DPN)-dependent system, and that the aerobic phosphogluconate pathway was absent. Although a DPN-linked lactic dehydrogenase was present, glucose was not attacked anaerobically. I postulate that pyruvate is removed preferentially by the pyruvic oxidase system and is not available for reduction to lactate, and that there is no alternative mechanism for the reoxidation of reduced DPN (DPNH). When the pyruvic oxidase system was poisoned by arsenite, cells catalyzed a homolactic glucose fermentation or, when an alternative mechanism for the reoxidation of DPNH was provided, such as the reduction of acetaldehyde by alcohol dehydrogenase, frozen and thawed cells attacked glucose anaerobically. Nevertheless, large amounts of lactate may be formed in nonaerated cultures. An understanding of the regulating mechanisms involved doubtless will be important in the study of nutrition.

Glycerol and lactate were oxidized to the same end products. Catalase is required for glycerol oxidation. This suggests that the initial stages might be the same as those shown by Gunsalus and Umbreit[4] for glycerol fermentation in some strains of *Streptococcus faecalis*, that is, phosphorylation to L-α-glycerophosphate, followed by oxidation by a flavoprotein to glyceraldehyde phosphate.

None of the intermediate compounds of the tricarboxylic acid cycle was metabolized. Of the amino acids, only serine and threonine were attacked, probably by dehydrases, the corresponding keto acids then being oxidized by the pyruvic oxidase system. Transaminases could not be detected. The terminal respiratory system catalyzing the transfer of electrons from DPNH to oxygen was not studied, but no evidence of the presence of a cytochrome system was observed. Enzymes degrading nucleic acids[5] and proteins (unpublished data) have been demonstrated.

Although incomplete, this picture shows that *M. mycoides* is restricted in its catabolic capabilities.

NUTRITION

Optical density has been used as an index of growth. It must not be assumed that with *M. mycoides* turbidity is always proportional to cell mass, since this will depend also on the size and shape of the elements, but increases in turbidity have been found to be paralleled by proportional increases in titratable acidity.

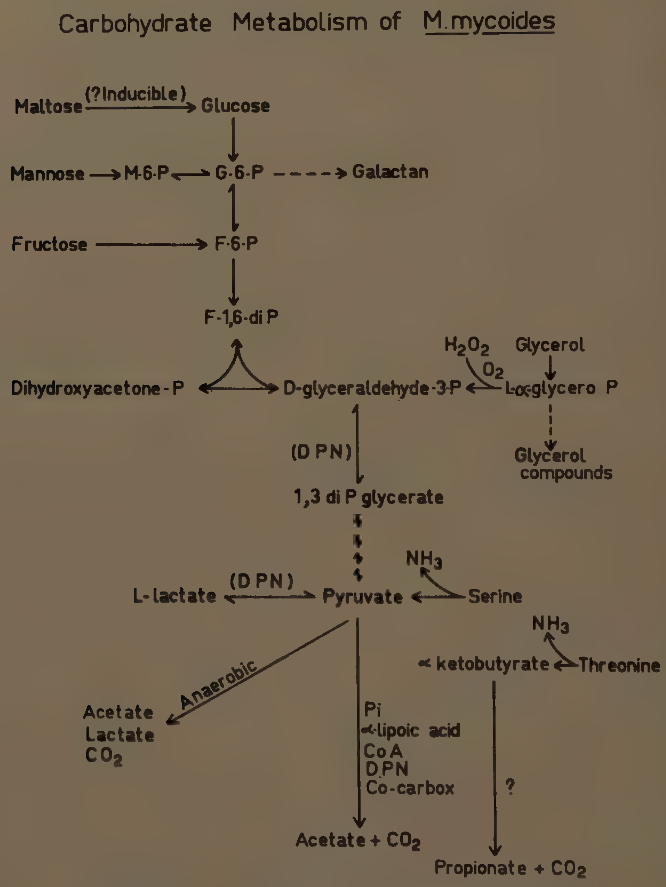

FIGURE 1. Carbohydrate metabolism of *M. mycoides.*

Replacement of Serum

Like all members of the Mycoplasmataceae except *M. laidlawii*, *M. mycoides* requires factors for growth that may be supplied by serum, ascitic fluid, or egg yolk. Edward and Fitzgerald[6] and Edward[7] already had shown the organisms' requirement for cholesterol, and Smith and his collaborators[8-11] have studied the lipid requirements of the PPLO.

A system replacing serum for growth of *M. mycoides* in a crude basal medium was described previously.[12] This consisted of a heat-stable defatted serum-protein fraction, cholesterol, and either Tween 80 or an unsaturated fatty acid in balanced proportions. The optimum concentrations of all three components were interrelated; an excess of either cholesterol or of fatty acid inhibited growth (FIGURES 2 and 3).

The serum fraction (fraction C) was prepared by a method that, although evolved independently, is somewhat similar to that used by Smith *et al.*[8-10] for their serum fraction B. As originally prepared, it contained approximately 4

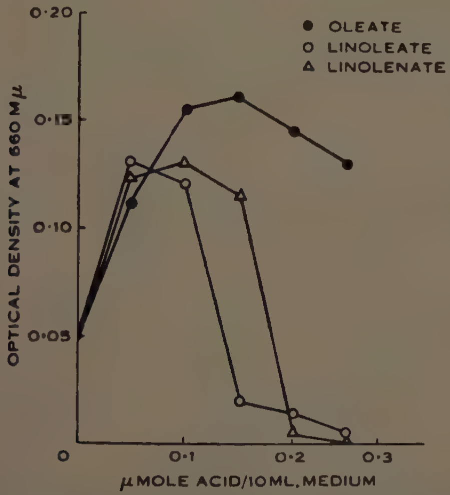

FIGURE 2. Effect of unsaturated fatty acids on growth of *M. mycoides*. Fraction C, 5 mg./10 ml. medium; cholesterol, 0.5 μM/10 ml. medium. Reproduced by permission from the *Australian Journal of Biological Sciences.*

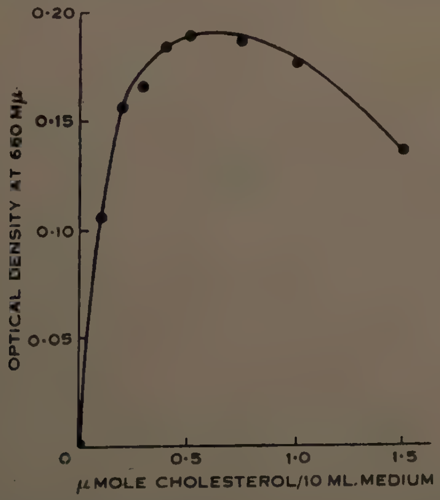

FIGURE 3. Effect of cholesterol concentration on growth of *M. mycoides*. Oleate, 0.15 μM/10 ml. medium; fraction C, 5 mg./10 ml. medium. Reproduced by permission from the *Australian Journal of Biological Sciences.*

per cent phospholipid, which was not essential. When phospholipid was removed, the optimum cholesterol concentration was reduced from 0.05 to 0.01 μM/ml. (for a concentration of 0.5 mg./ml. fraction C).

Cells of *M. mycoides* are very sensitive to lysis by surface-active substances. Fraction C or cholesterol inhibited lysis by oleate, and a mixture of both, in the proportions optimal for growth, was more effective than either alone (FIGURE 4). Thus, the function of the fraction C-cholesterol system seems to be protective, but only when the crude basal medium can be replaced by one of defined composition will it be possible to assess finally the lipid requirements.

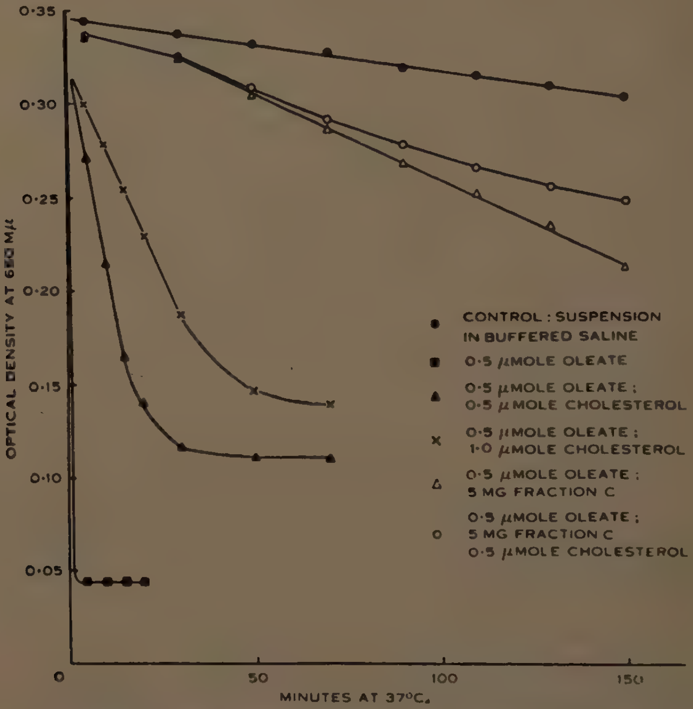

FIGURE 4. Inhibition of oleate lysis of buffered saline suspensions of *M. mycoides* by cholesterol and by fraction C. Mixtures of oleate and inhibitors incubated at 37° C. for 15 min. before system was completed by addition of cells. Additions per 10 ml. of medium were as indicated. Reproduced by permission from the *Australian Journal of Biological Sciences*.

Partly Defined Medium

A partly defined medium has now been evolved (TABLE 1). Its composition will be discussed briefly.

Albumin. With this medium, the addition of the components that replace serum in a crude medium would not give good growth from small inocula unless serum albumin also was added. With all these additions Tween 80 continued to stimulate growth, and higher concentrations could be tolerated. The addition of albumin did not affect the cholesterol requirement, nor could it replace fraction C.

Lactate. Earlier, in a crude medium, with serum replaced by the fraction C-cholesterol-Tween 80 system, a requirement for an osmotic pressure of about 12 atm. was recognized.[12] For this sodium acetate was used, but other salts or even sucrose would produce the same result.

In the partly defined medium, lactate in high concentration was required, L-lactate being more active than D-lactate (FIGURE 5). It was necessary to increase the concentration required with increasingly aerobic growth conditions. FIGURE 6 shows the growth responses to varying concentrations of sodium DL-lactate when the ionic strength was kept constant with sodium acetate in 18-mm. tube cultures incubated in air under 3 conditions: (1) standing undisturbed, culture volume 10 ml.; (2) standing undisturbed, culture volume 5 ml.; and (3) culture volume 5 ml. in tubes incubated in a rack inclined at an angle of 20° from the horizontal and rotating at 10 rpm. Thus, while high concentrations of L-lactate permit aerobic growth in the partly defined medium, crude supplements are effective in much lower concentrations. Attempts are being made to identify the active components of these complex supplements.

Table 1

Composition of Partly Defined Medium. Glucose and Bovine-Serum Albumin Added from Sterile Solutions After Autoclaving Medium

Composition of partly defined medium			
Component	Amount/l.	Component	mg./l.
Na_2HPO_4-KH_2PO_4, pH 7.8	0.04 M	Adenine	5.0
Sodium DL-lactate	0.15 M	Guanine	5.0
Glucose	5.0 gm.	Uracil	5.0
Bovine-serum fraction C	0.5 gm.	Thymine	2.5
Bovine-serum albumin	2.0 gm.	Ca pantothenate	0.25
Cholesterol	10 μM	Riboflavin	0.25
Tween 80	5.0 mg.	Pyridoxal-HCl	0.05
Glycerol	20 mg.	Nicotinamide	0.5
Acid-hydrolyzed casein	2.5 gm.	Thiamine	0.5
Tryptic digest of casein	2.0 gm. casein	Biotin	0.01
L-Cystine	2.5 mg.	DL-α-Lipoic acid	0.01
$MgSO_4 \cdot 7H_2O$	50 mg.		
$FeSO_4 \cdot 7H_2O$	0.25 mg.		
$MnSO_4 \cdot 4H_2O$	0.25 mg.		

Glucose. The function of the lactate is not simply to supply an energy source, as very little is metabolized; glucose or other metabolizable saccharide is indispensable. A galactan comprising about 10 per cent of the dry weight of the cells has been described by Plackett and Buttery.[13] Labeled glucose was shown to be a precursor of the polysaccharide (Plackett, unpublished data). Only a small fraction of the glucose carbon was incorporated, most of the sugar being catabolized. Glucose may be replaced for growth by maltose or, less effectively, by mannose or fructose, but not by galactose.

Glycerol. In early experiments, using a partly defined medium, there was no growth unless a fraction of Bactotryptone was added. This led to the isolation and identification of glycerol as the active component. As with lactate, greater concentrations were required with increasingly aerobic growth conditions. Growth responses to glycerol in cultures incubated in air under the same 3 growth conditions described for the lactate experiment are shown

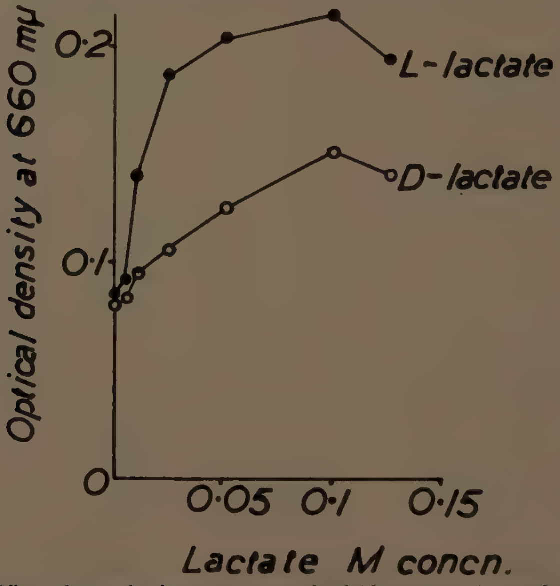

FIGURE 5. Effect of L- and D-lactate on growth of *M. mycoides* in partly defined medium Ionic strength was adjusted to 0.125 by additions of sodium acetate.

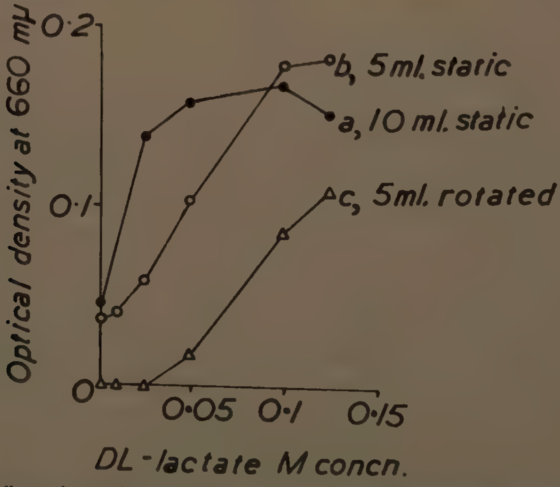

FIGURE 6. Effect of aeration on lactate requirement for growth of *M. mycoides* in partly defined medium. Ionic strength adjusted to 0.125 with sodium acetate. (*a*) Culture volume 10 ml., undisturbed; (*b*) culture volume 5 ml., undisturbed; (*c*) culture volume 5 ml., tubes inclined at 20° from horizontal and rotated at 10 rpm.

(FIGURE 7). The concentrations required for maximum growth were about 6, 10, and 30 μg./ml. under growth conditions 1, 2, and 3, respectively.

It was suggested earlier that glycerol oxidation might proceed via a flavoprotein-catalyzed oxidation of glycerophosphate. If this pathway were irreversible, glycerophosphate could not be formed from hexose, and an exogenous source of glycerol would be needed for lipid synthesis. Preliminary experiments have shown that C^{14}-labeled glycerol is incorporated into lipids by growing cultures, but also into a fraction that resists extraction with hot 5 per cent trichloroacetic acid (TCA) but becomes soluble in TCA after treatment with alkali, resembling in this respect the glycerophosphoprotein complex described in *Staphylococcus aureus* by Mitchell and Moyle.[14] This complex is thought to form part of the protoplast membrane.[15] Analyses of protoplast membranes by Gilby *et al.*[16] have shown the presence of glycerophosphate residues. Armstrong *et al.*[17] have described two cytidine nucleotides in *Lactobacillus arabinosus*. One of these, cytidine diphosphate glycerol, they believe to be the precursor of a glycerophosphate polymer.

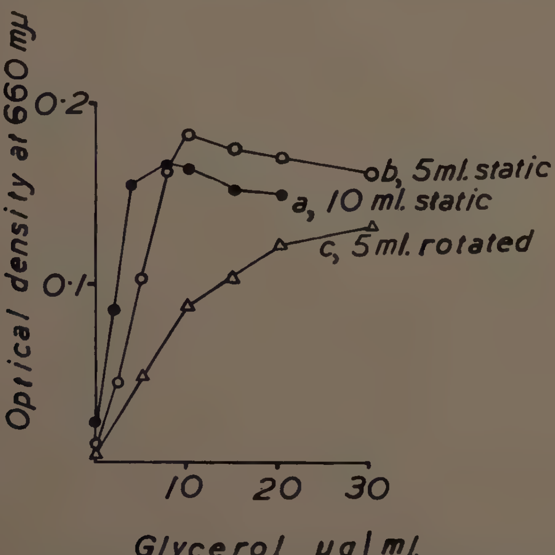

FIGURE 7. Effect of aeration on glycerol requirement for growth of *M. mycoides* in partly defined medium. (*a*) Culture volume 10 ml., undisturbed; (*b*) culture volume 5 ml., undisturbed; (*c*) culture volume 5 ml., tubes inclined at 20° from horizontal and rotated at 10 rpm.

Requirements for nucleic acid precursors and growth factors. With the use of serum albumin, lactate, and glycerol, as described, good growth can be obtained in the absence of crude supplements, but the possibility that the protein preparations may be supplying unspecified growth factors requires investigation.

The nucleic acid-precursor requirements could be supplied by a mixture of the free bases. Results of single-omission tests, using the V5 strain and a strain (GY) isolated from a goat,[18] are shown (TABLE 2). Guanine, uracil, and thymine proved essential, and the omission of adenine decreased growth by about one half. Cytosine was not required, and it could not replace any of the other bases. Guanine or uracil could not be replaced by hypoxanthine or orotic acid, respectively. The free bases could be replaced by the corresponding nucleosides or 2′,3′-nucleotides. No pentose was required.

As growth factors, riboflavin, thiamine, and nicotinamide (or nicotinic acid) were required. Omission of α-lipoic acid was without effect on growth in the presence of amorphous bovine-serum albumin (fraction V), but growth was decreased by about one half in the presence of crystalline albumin. The participation of α-lipoic acid in the pyruvic oxidase system was inferred from the sensitivity of the system to inhibition by arsenite. Leucovorin and pyridoxal are probably not required, at least in this medium.

Table 2

Nucleic Acid-Precursor and Growth-Factor Requirements of *M. mycoides*, Strains V5 and GY

Component omitted	Strain		
	V5		GY
	Expt. 1	Expt. 2	Expt. 3
Guanine	0	0	3
Uracil	0	0	0
Thymine	0	0	3
Adenine	57	56	57
Cytosine	105		
Riboflavin	3		13
Thiamine	17		50
Nicotinamide	5		0
DL-α-Lipoic acid	95	56	60
Biotin	100	103	53
Pantothenate	96	99	58
Leucovorin	89		
Pyridoxal	85	87	98

Key: Expt. 1, growth in the presence of bovine-serum albumin (BSA) fraction V (Armour). Expts. 2 and 3, BSA fraction V replaced by crystalline BSA (Armour).

Growth expressed as percentage of that in complete medium.

The GY strain had a partial dependence on biotin and pantothenate, in addition to the other requirements.

Amino acid requirements. The amino acid requirements have not been determined as yet, since it has not been possible to obtain growth in the absence of an enzymatic casein digest. Acid-hydrolyzed casein may be replaced by an amino acid mixture. Amino acid antagonisms are suspected, since growth inhibitions have been observed when the concentration of several of them was increased. It seems safe to predict from metabolic studies that the organism is exacting in its amino acid requirements.

This work has shown that *M. mycoides*, besides being restricted in its catabolic capabilities, is also relatively exacting in its nutritional requirements, reflecting limited biosynthetic powers.

The sensitivity of the PPLO to the physicochemical environment warrants further investigation. Undoubtedly this is related to lack of a rigid cell wall, absence of bacterial cell-wall "mucocomplex,"[19,20] and their cell-surface properties.

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NUTRITIONAL REQUIREMENTS OF PPLO AND THEIR RELATION TO METABOLIC FUNCTION*

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One of the distinguishing characteristics of the so-called parasitic pleuropneumonialike organisms (PPLO) is their requirement of a lipoprotein for growth. Although the saprophytic strains can be cultivated in the absence of a serum supplement, the growth requirement for sterol supplied by the serum or the lipoprotein has been used as a basis for classifying PPLO as a distinct group of microorganisms. The sterol requirement is not unique in nature, since some protozoa and the larval or pupal stages of several insects display a similar requirement. However, with most of the insects the sterol requirement is lost upon development to the pupal or adult stage. The function of the sterol in insects, although not clarified, appears to be to supply a precursor for synthesis of hormone-type steroids. The function of the sterol in protozoa is unknown.

PPLO offer an admirable system for the study of sterol function. Admittedly, the protozoa can be obtained in larger amounts, but most of those that require sterol cannot be propagated in pure culture. A paucity of catabolic mechanisms and the absence of a rigid cell wall are added advantages in the PPLO.

The nutritional studies conducted in our laboratory which culminated in the production of a minimal defined medium indicated that, aside from the lipoprotein requirement, nothing distinct could be expected from a study of the relation of a given required nutrient and its function that could not be obtained by a study of the same nature in a species of microorganism more adaptable to enzymic experimentation. This report is therefore restricted to the study of the lipoprotein, its requirement, and function.

Reports from our laboratory and others, in particular from Edward,[1] have shown that the serum requirement could be distinguished as a threefold requirement, that is, protein, sterol, and phospholipid or acetone-insoluble lipid. For the sake of completeness and clarity, a fourth component, fatty acids, will be included.

The Protein Moiety

A protein that was found to resemble the alpha-1 lipoprotein of mammalian serum was isolated by means of ammonium sulfate, heat, and acid precipitation. This protein contained the growth-promoting activity of serum.[2] Electrophoretic and sedimentation analyses revealed it to be a single pure entity. The properties of this purified protein, B-II, are shown in TABLE 1. Its electrophoretic mobility, sterol content, and isoelectric point are identical to those

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of the alpha-1 lipoprotein.[3] The lower sedimentation constant and molecular weight reflect the loss of phospholipid during the fractionation procedures. Tryptophan, leucine, valine, alanine, arginine, glutamic acid, glycine, and lysine were found to be the only amino acids comprising B-II.[4] As judged from the size and intensity of the Ninhydrin-positive areas, the predominating amino acids were leucine, arginine, glycine, and lysine, the latter being present in the greatest amount. The predominance of the amino acids lysine and arginine would indicate that the protein is basic in nature. This basicity undoubtedly imparts to the protein a characteristic necessary for its function.

The protein requirement may be said to be partially specific. Not every protein is capable of supporting growth; among many different proteins tested, only serum albumin and beta lactoglobulin were active in addition to B-II. On a quantitative basis these two proteins were only one tenth as active as B-II. Degradation of these proteins by enzymatic, acid, or alkaline hydrolysis resulted in complete destruction of activity.

TABLE 1
PROPERTIES AND CONSTITUTION OF FRACTION B-II AND ALPHA-1 LIPOPROTEIN*

Characteristics	B-II	Alpha-1 lipoprotein
Percentages of plasma protein	3	3
Percentage of free cholesterol	trace	none
Percentages of bound cholesterol	7.6	present
Percentages of phospholipid	0.07	present
Electrophoretic mobility	7.98 × 10 U./sec./v/cm. (similar to alpha-1 globulin)	similar to alpha-1 globulin
Isoelectric point	5.2	5.2
Sedimentation constant S_{20}	3.75	5
Molecular weight†	1.35×10^5	2×10^5

* Data from Cohn *et al.*[14]
† Molecular weight by osmotic-pressure measurement.

Since hydrolysis of a protein resulted in inactivation, it was unlikely that the PPLO would degrade the protein. Analyses run on culture supernatants showed no significant increase in free amino residues. However, approximately 0.3 mg./ml. of B-II disappeared during growth. This loss could be accounted for by uptake or adsorption onto the cells. Elucidation of the fate of the protein moiety must await experiments employing isotopic tracers.

The basic nature of active proteins prompted an examination of their comparative abilities to bind cholesterol. All proteins were rendered lipid-free prior to the experiments. The results showed that B-II bound about 34 moles cholesterol per mole of protein, while the values for bovine serum albumin and beta lactoglobulin were 9 and 8, respectively. Inactive proteins such as bovine gamma globulin failed to bind any cholesterol. The four- to fivefold greater cholesterol-binding capacity of B-II, as compared to the albumin and lactoglobulin, paralleled the approximately tenfold better growth-promoting activity of B-II.

Although much more is to be learned concerning the properties and capabilities of the protein moiety, it is of interest to speculate as to its function in light of the known facts.

It is possible to ascribe to the protein a function of detoxification of unsaturated fatty acids, since proteins are known to perform this activity in other microorganisms. Employing 3 toxic levels of caprylic and oleic acids (5 to 15, and 0.1 to 1.0 mg./ml., respectively), the addition of varying levels of B-II had no detoxifying effect, whereas the crude serum fraction and lipid-free bovine serum albumin did reduce the inhibition. It appears obvious that a protein capable of supporting growth is intimately related to the lipid requirements. It is probable that the protein required for growth either facilitates exchange of water-insoluble lipids with cellular lipoproteins or is incorporated as a cell lipoprotein.

The Sterol Component

The second requirement under consideration is the sterol component. Edward and Fitzgerald[1] reported that cholesterol was active in promoting growth and could be replaced with either cholestanol or stigmasterol, whereas ergosterol, coprostanol, and certain cholesteryl esters were inactive. A previous report from this laboratory indicated that of 5 cholesteryl esters tested, only cholesteryl laurate was active. As an aid to elucidating the function of the sterol component, a study of the quantitative response to various sterols was undertaken.[5] An effort was made to select sterols possessing certain modifications of the molecular configuration of cholesterol. Cholesteryl esters of fatty acids were not available commercially and therefore were synthesized by the method of Swell and Treadwell.[6] The esters were then purified in order to free them of cholesterol contamination, as judged by the absence of a precipitate upon addition of digitonin and by melting-point values. The test medium was rendered lipid-free by ether extraction and consisted of PPLO broth[7] plus methanol-extracted B-II at a level of 0.5 mg./ml., and lecithin at 0.005 mg./ml. A negative control containing no sterol and a positive control consisting of unextracted medium and B-II were run.

Initially, a dose response curve was constructed for cholesterol. From this it was noted that the optimum level of cholesterol constituted 0.01 to 0.02 mg./ml. or 3 to 6 $\times$ 10^{-5} M. Concentrations of other sterols tested were kept within this range. TABLE 2 lists the growth response to active sterols. A value of 1.00 is equivalent to growth obtained with the positive control. Only 2 compounds, β-sitosterol and cholestanol, adequately replaced cholesterol.

Comparing the molecular structure of cholesterol to these, it can be noted that cholestanol is the unnatural 5α-reduced cholesterol. The natural 5β compound, coprostanol, is inactive, while β-sitosterol has a ring structure identical to cholesterol, but with an extra 2 carbons on the side chain. Among the less active sterols, stigmasterol is identical to β-sitosterol, with the exception of a double bond at carbon 22; ergosterol differs from cholesterol by having an additional ring double bond at C-7, another double bond at C-22 (as stigmasterol), and an extra carbon atom on the side chain. Epicholestanol differs from cholestanol in that the 3-OH group is α rather than β. The cholesteryl esters are self-explanatory. Thus it can be seen that even minor modifications of the cholesterol molecule reduce activity. More drastic changes such as removal of the side chain, aromatization of ring A, oxidation of the 3-OH to a keto group, reduction to the hydrocarbon, or formation of an ether linkage at the 3-OH group completely inactivate the molecule. Indeed, some of these changes impart an inhibitory activity to the molecule (TABLE 3). The hydrocarbons, cholestane, and the bicholesteryl ether, as well as the 2 precursors, mevalonic acid and squalene, were inhibitory. These results indicated that the sterol requirement is specific, not only for the 3-OH group and the length of the side chain, but for the degree of unsaturation in both ring and side chain.

TABLE 2

GROWTH RESPONSE OF STRAIN O7 TOWARD VARIOUS STEROLS

Sterol	Concentration* (M)	Growth response†
None	—	0.01–0.04
Cholesterol	10^{-5}	1.00
Cholesteryl butyrate	10^{-5}	0.53
Cholesteryl valerate	10^{-5}	0.48
Cholesteryl laurate	10^{-5}	0.28
β-Sitosterol	3×10^{-5}	1.16
Stigmasterol	1.5×10^{-4}	0.22
Ergosterol	10^{-5}	0.68
Cholestanol	3×10^{-5}	2.06

* Molar concentration giving best growth response.
† Relative growth compared with cholesterol.

TABLE 3

INHIBITORY ACTIVITY OF VARIOUS STEROLS ON GROWTH OF STRAIN O7

Compound	Concentration (M)	Growth response*
No sterol		0.01–0.04
Unextracted medium		1.00
Squalene	3×10^{-5}	0.003
Mevalonic acid	3×10^{-5}	0.002
Cholestane	3×10^{-5}	0
	1.5×10^{-4}	0
Bicholesteryl ether	3×10^{-5}	0.007

* Relative growth compared with unextracted medium.

Cholesterol uptake by the cells during growth can be demonstrated. As described by Lynn elsewhere in this monograph, there occurs a logarithmic uptake per milligram of cell N during the log phase of growth. The nature of the sterol appears to be unchanged in the organism. At least no degradation, as judged by disappearance of cholesterol, occurs. Chromatography of alcohol-ether extracts of cells or purified sterol shows the presence of cholesterol only. Compounds representing the next oxidized or reduced states of cholesterol were not evident.

The absolute requirement of the 3-OH group and the activity of certain fatty acid esters of cholesterol could be indicative of an enzymatic function at this site. As shown in TABLE 4, those strains that require the lipoprotein factor are capable of hydrolyzing cholesteryl esters of fatty acids, while the saprophytic strains possess very weak activity. Growth of the saprophytes in the presence of lipoprotein did not affect their activity. The enzymatic nature of the reaction was confirmed by demonstration that increased cell protein proportionately increased activity and that heating destroyed activity. The reaction resulted in the appearance of equimolar amounts of free cholesterol and fatty acid. Sonicated cells retained all the activity of whole cells. Although dialyzed sonicated cells possessed no absolute cofactor requirement, many surface-active compounds stimulated the liberation of free cholesterol (TABLE 5). The optimum level of lecithin or cholate was a ratio of 1:1 with ester. Raising this level had no effect. The nonspecificity of these compounds indicates a solubilizing action on the sterol.

TABLE 4

CHOLESTEROL ESTERASE ACTIVITY OF VARIOUS STRAINS OF PPLO

Strain	Cholesteryl ester						
	Propionate	Butyrate	Valerate	Caprylate	Laurate	Palmitate	Oleate
	Free cholesterol formed per milligram N in 6 hours (μM)						
O7	0.54	1.38	0.84	1.02	1.08	0.84	1.02
39	1.74	1.14	0.42	1.62	1.74	—	—
48	—	2.86	1.92	2.16	0.96	—	—
J	—	2.58	1.20	1.62	0.42	—	—
Campo	0	0.12	0.22	0	2.09	0.25	0.19
B-15	0	0	0	0	0	0.05	0.06
Laidlaw B	0	0.17	0.12	1.62	0	0	0

TABLE 5

EFFECT OF SURFACE-ACTIVE COMPOUNDS ON HYDROLYSIS OF CHOLESTERYL VALERATE BY STRAIN O7

Cofactor	Free cholesterol formed per milligram N in 6 hours (μM)
None	1.12
Lecithin, 1 μM	1.26
Lecithin, 10 μM	1.53
Lecithin, 20 μM	1.55
Na cholate, 10 μM	1.57
Cephalin, 10 μM	1.49
Tween 80, 0.01%	0
Soap, 0.01%	1.34

The synthesis of cholesteryl esters from free cholesterol and fatty acids was shown to occur with whole cells, but to a much smaller degree than with hydrolysis (TABLE 6). Since the equilibrium of the reaction might favor hydrolysis, emphasis should be placed on the fact that synthesis does occur rather than on quantitative results. Sodium taurocholate was shown to be required for synthesis by whole cells.

It was of interest to investigate the effect of coenzyme A on the reaction, since it is intimately involved in fatty acid oxidation. In regard to synthesis of cholesteryl esters, it could be required to activate fatty acid prior to its coupling with sterol. Breaking of the ester bond in the presence of coenzyme A and ATP might conceivably occur by thiolytic cleavage. TABLE 7 shows that coenzmye A, in conjunction with ATP, is an absolute requirement for the synthetic reaction and is stimulatory for the hydrolytic reaction. This effect could be noted only in preparations freed of coenzyme A by Dowex treatment. The relatively poor activity is due probably to the small amount of enzyme present, since each mixture contained only the remainder of 1 mg. of cell N measured prior to preparation. The possibility that an activated fatty acid was formed following hydrolysis of ester was tested by analyzing the final reaction mixture for acyl coenzyme A and by incorporating hydroxylamine into the reaction mixture to trap activated acyls. It was found that acyl coenzyme A is detectable in amounts greater than the cell controls. Hydroxylamine had a stimulatory effect on the hydrolytic reaction.

TABLE 6

SYNTHESIS OF CHOLESTERYL ESTERS BY STRAIN O7

Fatty acid	Ester formed per milligram N in 6 hours (μM)
Acetic	0.30
Propionic	0.13
Butyric	0.42
Valeric	0.20
Lauric	0.12

TABLE 7

EFFECT OF COENZYME A AND ADENOSINETRIPHOSPHATE (ATP) ON SYNTHESIS AND HYDROLYSIS OF CHOLESTERYL ESTERS BY STRAIN O7

Esters	No addition	Coenzyme A + ATP
	Ester (μM)	
Synthesis		
Cholesteryl butyrate	0	0.20
Cholesteryl caprylate	0	0.30
	Cholesterol (μM)	
Hydrolysis		
Cholesteryl butyrate	0.33	0.56
Cholesteryl caprylate	0.55	0.99

When the rates of hydrolysis of 2 esters at pH 6.5 were determined, a typical second-order reaction occurred up to 4 hours, when renewed release of fatty acid began. Allowing the reaction to proceed at pH 7.5, the pH at which fatty acids are known to be oxidized by these organisms, should result in no accumulation of fatty acid and in a first-order type of rate curve, since rapid removal of fatty acid would tend to drive the esterase reaction toward hydrolysis. Experimentation proved that no appreciable fatty acid accumulation occurred, but that a second-order rate curve persisted for free-sterol accumulation.

An attempt was made to define the cellular site of esterase activity. When the esterase activity of residue (cell membrane) and supernatant (cell protoplasm) fractions (TABLE 8) was assayed, it was found that almost all the activity was associated with the residue.

The specificity of the sterol growth requirement prompted an examination of the specificity of the esterase. The use of esters of other sterols containing the 3-β-hydroxy group, aliphatic esters, and neutral fats as substrates revealed that there was weak activity with the acetates of stigmasterol and β-sitosterol, no activity with aliphatic esters, but considerable activity toward neutral fats. Thus it was necessary to determine whether the esterase and lipase activities were distinct or whether the esterase activity was a manifestation of the lipase. As with the esterase, lipase activity was associated almost exclusively with the residue fraction. On the other hand, the 2 activities behave quite differently in regard to lability to heat and *p*H and to the inhibitory action of various compounds. The lipase was more stable to heat and sterol inhibitors, but less stable to *p*H change than the esterase.

TABLE 8
CHOLESTEROL ESTERASE ACTIVITY OF VARIOUS CELLULAR FRACTIONS OF STRAIN O7

Cell fraction	Cholesteryl propionate	Cholesteryl valerate
	Free cholesterol formed per milligram N in 6 hours (μM)	
Whole cells	0.95	1.50
Sonicated cells	1.32	1.20
Residue	1.06	0.95
Supernate	0.11	0.40

TABLE 9
EFFECT OF POSSIBLE INHIBITORS ON HYDROLYSIS OF CHOLESTERYL VALERATE BY STRAIN O7

Inhibitor	Free cholesterol (μM)	Inhibition (per cent)
None	4.10	—
Cholestane, 10^{-3} M	0	100
Cholestane, 10^{-4} M	0.10	98
Bicholesteryl ether, 10^{-3} M	0	100
Bicholesteryl ether, 10^{-4} M	0.09	98
2,4-dinitrophenol, 10^{-4} M	2.18	47

TABLE 9 shows the inhibitory effect on the esterase of several compounds. It is interesting that, paralleling their inhibitory action on growth, cholestane and bicholesteryl ether inhibit esterase activity. Their inhibitory action undoubtedly is associated directly with cholesterol. Digitonin probably inhibits by irreversibly binding free cholesterol, and 2,4-dinitrophenol may act indirectly by interfering with fatty acid oxidation, resulting in fatty acid accumulation and, thus, a shifting of the equilibrium toward synthesis of ester.

If these data on the nutritional requirement of sterol and on the enzymatic activity relative to cholesterol are kept in mind, it would be opportune to analyze the function of the sterol. Generally, it could be assumed that cholesterol could function in these organisms by detoxifying fatty acids, by providing an oxidizable substrate, by maintenance of the structural integrity of the cell, or by involvement in the permeation of substrates.

Cholesterol could, by formation of its esters, bind fatty acids and slowly release them in the required nontoxic amounts. Studies to date, although incomplete, have not revealed a requirement for unsaturated fatty acids. Saturated fatty acids, which may act as the carbon and energy source for most human strains, are required, but are relatively nontoxic; that is, in levels in some instances as high as 10 mg./ml. When toxic levels of either unsaturated or saturated fatty acids are added to the growth medium, cholesterol alone or cholesterol plus lecithin—no matter what level is present—fail to reduce the inhibitory action. If cholesterol were to function as a detoxifier, one would expect to observe some diminution of the inhibitory action of fatty acids in this experimental situation.

Reports of chemical analyses of mitochondria capable of carrying out oxidative phosphorylation have made known the presence of cholesterol and its esters. Present evidence and opinion point to the rather negative role of sterol as a component of a lipoprotein matrix for the active water-insoluble components of oxidative phosphorylation. However, some evidence contradicts this assumption. Employing *Tetrahymena pyriformis*, Conner[8] has shown that growth inhibition caused by 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, can be reversed by stigmasterol. This would indicate a direct function of sterol in oxidative phosphorylation. Talalay[9] has shown that steroids can function in the transhydrogenase reaction. Thus estrone accepts H^+ from reduced TPN to form estradiol-17β, which in turn yields the H^+ to DPN. Talalay visualizes other steroid hormones functioning similarly and, by doing so, regulating the physiology of the organism by favoring hydrogenation (synthesis) or phosphorylation (degradation), depending upon the abundance of a particular steroid dehydrogenase present. Thus the presence of an overabundance of H^+-accepting steroid would result in the shifting of transhydrogenase equilibrium toward reduced DPN and hence to phosphorylation. Although it is not impossible that cholesterol (by virtue of its double body at C-5) and its 3-OH group can be alternately oxidized and reduced, no evidence of transformation of cholesterol to cholestanol has been obtained. The natural reduction product of cholesterol is coprostanol, the 5β compound. However, small amounts of cholestanol occur naturally with cholesterol. Oxidation of the 3-OH group of cholesterol to give 4-cholesten-3-one has been shown to occur in bacteria.

In regard to PPLO, some preliminary probes have been made to detect some relationship between sterol requirement and oxidative phosphorylation. Experiments were done in an attempt to demonstrate growth inhibition by 2,4-dinitrophenol similar to that reported for *Tetrahymena*. No growth inhibition was observed on a quantitative basis even to saturation levels of 2,4-DNP. This may have been a wrong approach since it would be necessary for any inhibition, if it could be relieved by cholesterol, to occur in the presence of considerable sterol in the growth medium. A better approach is the utilization of an isolated system that carries out oxidative phosphorylation. Such a system might be that of monohydric alcohol or acetate oxidation. Needless to say, nothing has been accomplished to rule out a function for cholesterol in oxidative phosphorylation. That cholesterol might serve as a carbon and energy source for PPLO is another possibility. Some bacterial species are capable of complete oxidation of cholesterol. The addition of cholesterol in substrate amounts to growing or resting cells of PPLO resulted in neither O_2 uptake nor detectable loss of total sterol. Indeed, substrate levels inhibited endogenous respiration. The addition of various cofactors and of thyroxine, said to stimulate cholesterol oxidation, has no effect. Therefore, it is very unlikely that cholesterol performs such a function in PPLO.

As was mentioned in the discussion of oxidative phosphorylation, cholesterol may serve as a component of the lipoproteins of mitochondria, whose function may be solubilization of water-insoluble lipids. Cholesterol can also be a component of the lipoprotein of the cell membrane. In this instance, too, it may aid, in conjunction with the protein moiety, in solubilization of lipids directly involved in electron transport, for Mitchell has postulated that the initial and terminal enzymatic reactions involved in substrate degradation and, thus, electron transport, occur at the lipoprotein-containing cell membrane of microorganisms. An evaluation of this function must necessarily await elimination of other possible functions.

The last function for sterol to be considered is that of substrate permeability. First, let us assume as a working hypothesis that cholesterol present in the cell membrane is esterified by the mediation of the cholesterol esterase and that the cholesteryl ester is then made accessible to the protoplasm, whereupon it is hydrolyzed to liberate free or active fatty acid.

What evidence is there to support such a theory? As reported in this monograph by Lynn, cholesterol is found in the cell membrane predominantly in the ester form but, in the protoplasm, predominantly as the free form. The fatty acid components of the esterified cholesterol are almost exclusively volatile acids. Almost all of the cholesterol esterase activity is located in the cell membrane. Such a distribution of ester and free cholesterol and of cholesterol esterase would be expected by the above hypothesis. The specificities of the sterol growth requirement and the cholesterol esterase are parallel. Only those strains of PPLO requiring the lipoprotein growth factor and containing cholesterol possess the ability to oxidize fatty acids. The probable thiolytic cleavage of an ester to yield acyl coenzyme A, which is the initial intermediate in fatty acid oxidation, and the stimulation of the reaction by the removal of activated acyls through the action of hydroxylamine favor a mechanism of fatty acid permeability.

On the other hand, one would expect better esterase activity if fatty acids were the sole oxidizable substrate. The poor activity demonstrable by supplying cholesteryl esters may not be a true measure of total activity. The mechanism visualized for permeation of fatty acids would utilize sterol already present in the cell in soluble form by virtue of its complexing with protein. Since sterol is not degraded, the fraction of added sterol capable of being solubilized and thus utilized for fatty acid transport would be dependent upon the remaining available sites on the protein and would probably be small. Therefore, some other method should be devised.

The permeability of a metabolizable substrate has been shown by Cohen and Monod[10] and by Mitchell[11] to be due in many instances to some enzymatic mechanism located at the cell surface. In the case of fatty acids and PPLO, the cholesterol esterase could function in such a manner. The possibility that this esterase functions as a permease for sterol can be discounted at present by the fact that growth and sterol uptake occur in the absence of fatty acid, and by the findings of Swell *et al.*[12] that bile acids, but not fatty acids, are required for incorporation of cholesterol C^{14} into the intestinal wall of rats. Additional evidence that sterols may be involved in permeability has been reported by Lester *et al.*[13] Briefly, these investigators have noted the fact that desoxycorticosterone inhibits the uptake of sugars, amino acids, and rubidium by *Neurospora crassa.* This inhibition of uptake could be reversed by cholesterol, ergosterol, corticosterone, and 17α-hydroxy desoxycorticosterone. Upon further study of rubidium uptake, it was shown that the process could be divided into two phases, one being active transfer into the cell, which is nonspecific, the other being a binding event responsible for the accumulation of rubidium, which is specific and is characterized by a sensitivity to desoxycorticosterone.

The hypothesis that cholesterol esterases and cholesterol function in fatty acid transport is not a new one. At one time this was the favored explanation of esterase function in mammalian systems. Now it is felt that serum albumin–complexed fatty acid serves as the transport mechanism. Indeed, there is a rapid exchange of fatty acid through the mediation of serum albumin in mammalian systems. Substitution of the lipoprotein with lipid-free bovine serum albumin at a concentration of 10^{-2} M together with various fatty acids in ratios of fatty acid to albumin of 1.5 to 7 failed to support growth. This result would indicate that such a transport mechanism does not operate in PPLO or that the hypothesized function of the lipoprotein is incorrect.

Hence we have evidence that favors a function for sterol either in oxidative phosphorylation or substrate permeability. The latter appears to have the preference at present; indeed, the sterol may be involved in permeability mechanisms far broader than the fatty acid permeability discussed for PPLO.

The Phospholipid Component

The term phospholipid requirement, as will be seen, is not a strictly accurate one. It has been reported from this laboratory that lecithin was a growth requirement for certain PPLO. The level of this compound that permitted growth was very critical, it being necessary to maintain a ratio of lecithin to cholesterol of 0.5 or less.[3] Higher ratios resulted in growth inhibition. Edward and Fitzgerald[1] found that an acetone-insoluble lipid fraction of egg yolk satisfied the requirement, but that a lecithin, cephalin, and sphingomyelin were nadequate. A quantitative analysis of the growth response to various compounds that might satisfy this requirement showed that lecithin and cholate at ratios to cholesterol of 0.5 were adequate. Cephalin was inactive, as were the component parts of a lecithin (that is, fatty acids, gylcerol, and choline or 2-aminoethanol). With respect to molecular structure, lecithin and cholate are vastly different compounds. However, when the biological properties of the 2 compounds are examined, 1 common property, surface tension depression, is observable. Although several other surface-active compounds are yet to be tested for growth-promoting activity, it seems clear that a surface tension depressant is what is required.

Lecithin does not appear to be synthesized. Similarly, the addition of lecithin as a substrate to resting and growing cells results in no liberation of phosphate or fatty acid.

On the other hand, both lecithin and cholate, with other surface-active compounds, were shown to act as stimulatory cofactors in the cholesterol esterase reactions. Lecithin, alone or in combination with cholesterol or the protein or both, and at varying concentrations, fails to relieve fatty acid toxicity.

Two possible functions of this phospholipid present themselves. One is the solubilization of water-insoluble lipids, such as cholesterol, either by reducing surface tension or by acting as a link between the protein moiety of lipoprotein and the sterol. Lecithin is known to increase the water solubility of cholesterol and is also present in rather large amounts in lipoproteins. The second possible function is involvement in oxidative phosphorlyation, either by acting as a component of a lipoprotein matrix, as described elsewhere in this monograph, or by actually participating in electron transfer. Recent published data indicate that a quinone-type compound rather than a phospholipid functions in this latter capacity.

The Fatty Acid Component

In order to complete the over-all picture of lipoprotein growth requirement and function, it seems appropriate to mention the fatty acids. Excessive extraction of the basal growth medium with ether at *p*H 4 by necessity removes fatty acids. That some ether-soluble component other than cholesterol and lecithin was stimulatory for growth was noted in the growth experiments on the phospholipid requirement. Growth in this instance was not equivalent to the control. Thus nontoxic levels of saturated and unsaturated fatty acids were added to the medium to determine if optimal growth could be restored. Among nine fatty acids tested, only acetate restored growth to the optimal level. One milligram per milliliter was optimal. Since acetate might behave as a sparker for fatty acid oxidation, the effect on growth of a suboptimal amount of acetate together with butyrate was tested. Neither the suboptimal level of acetate nor the substrate amount of butyrate alone permitted optimal growth, but the combinations of the two compounds did. Combinations of acetate and other short-chain fatty acids gave similar results.

The enzymatic activity of PPLO with respect to fatty acids is achieved through at least three mechanisms. One is the participation of fatty acids in the esterase reaction; another is the participation in the lipase reaction. Of particular importance is the oxidation of fatty acids, discussed elsewhere in this monograph by Lynn. In connection with the last activity it appears that fatty acids constitute the major carbon and energy source of most human strains of PPLO. This probably constitutes their major function. The results of the growth experiments with suboptimal levels of acetate plus substrate amounts of other short-chain fatty acids indicate a sparking activity for acetate in fatty acid oxidation.

Conclusions

All these data tend to indicate two possible functions for the lipoprotein growth factor. Neither can be eliminated at this time. The two functions involve cellular permeability and oxidative phosphorylation. A mechanism for each of these functions can be postulated.

In regard to cellular permeability, the lipoprotein either is incorporated into the cell or, more likely, facilitates rapid exchange of the cholesterol with cell lipoproteins, the protein moiety acting as a site for attachment or as a solubilizer of cholesterol. Lecithin may act as a link attaching sterol to protein. The cholesterol, being neither lost nor formed, is utilized by the cholesterol esterase with the cofactor participation of lecithin and some high-energy compound for synthesis of esters from activated fatty acids. The esters are then transported across the cell membrane and hydrolyzed to liberate free fatty acid or acyl coenzyme A as an oxidizable substrate. The data supporting such a mechanism have been presented. However, certain discrepancies must be taken into consideration. If all strains of PPLO that require the lipoprotein for growth utilized fatty acids as the sole carbon source, this theory would appear more valid. Avian strains and some human strains, although capable of oxidizing fatty acids, do not depend upon these as the sole carbon source, but oxidize carbohydrates also. It may be that this proposed mechanism also supplies essential fatty acids and other compounds for all parasitic strains. Essential fatty acid requirements of all the various strains still require clarification. The results of Lester *et al.*[13] with *N. crassa* lead one to believe that sterol plays a broader function in permeability than merely the transport of fatty acid. It is not conceivable that the esterase would function in permeability of other components, as anions or cations. Indeed, additional work is required to prove or disprove this hypothesis.

In regard to oxidative phosphorylation, the lipoprotein could, as in the previous hypothetical mechanism, facilitate exchange of lipid with cell lipoproteins. The lipoproteins formed by acceptance of the sterol and phospholipid could act as a matrix for the water-insoluble components of the oxidative phosphorylation system. Although the cholesterol and lecithin might participate in electron transfer, it is unlikely. Cholesterol would appear to have an additional function because of the relatively large amounts contained in the cells. Additional data supporting and opposing such a function have been presented.

Finally, one asks the question, What significance has this lipoprotein requirement and function for PPLO and L forms, in general? Certainly, it is not common to all PPLO and L forms. Many L forms grow well without serum supplements. With many of these there appears to be a need for hypertonicity of medium. Such a need is not displayed by PPLO, and evidence indicates that neither the lipoprotein nor its components function by a detoxification effect. The saprophytic PPLO require neither serum nor a hypertonic medium. Experiments recently conducted at our laboratory with the Laidlaw B and B-15 strains have shown that quantitatively, growth in lipid-free medium is equivalent to growth in unextracted medium. However, addition of the PPLO serum fraction to large-volume cultures increases yields. Studies on the lipid composition of these saprophytic strains has not revealed so much as a trace of any sterol. There is, however, an alcohol ether–soluble yellow pigment in the two strains examined. What function the serum fraction plays in growth stimulation of the saprophytes needs further clarification.

The answer to the lipoprotein requirement and function may lie in the answer to the question of where PPLO arise or arose and the mechanisms of their development.

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HYSIOLOGICAL AND SEROLOGIC COMPARISONS OF PPLO FROM VARIOUS SOURCES*

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The relationship between pleuropneumonialike organisms (PPLO) and L orms of bacteria has been the subject of numerous reviews (Dienes and Wein- erger, 1951; Edward, 1954; and Klieneberger-Nobel, 1954). Wittler *et al.* 1956) and Smith *et al.* (1957) have demonstrated physiological and serologic imilarities between PPLO and *Corynebacterium* spp. That PPLO are L forms f bacteria, however, is not yet an accepted fact (Edward and Freundt, 1956).

With present techniques, numerous PPLO have been isolated. Many of these rganisms have been studied morphologically, pathologically, and serologically. Vith the exception of a few strains, little is known of their physiology and etabolism. Dujardin-Beaumetz (1900) identified acetic acid as the only olatile acid produced by *Mycoplasma mycoides* from carbohydrate. Holmes nd Pirie (1932) demonstrated lactic dehydrogenase in this organism. Rodwell nd Rodwell (1954) showed that cell suspensions of *M. mycoides* oxidized glu- ose and pyruvate aerobically to acetate and CO_2. Anaerobically, glucose as not utilized, and pyruvate was dismutated to lactate, acetate, and CO_2. mall amounts of acetylmethylcarbinol were also produced in the latter case. ricarboxylic acid cycle intermediates were not utilized. Metabolic similari- ies between *M. mycoides* and *Streptococcus faecalis* were noted.

Lecce and Morton (1954) showed that certain human strains do not utilize arbohydrates or tricarboxylic acid cycle intermediates. Active alcohol de- ydrogenases were observed in these organisms. Smith (1955, 1957*a*, 1957*b*, 957*c*), studying the amino acid metabolism of the same organisms, showed e phosphorolytic deamidation of glutamine to be the only reaction of amino cids capable of producing energy that could be utilized for growth.

In order to understand this group of organisms further, a physiological and rologic study of various strains was made. With the exception of four strains, ll organisms produced acid from carbohydrate. Metabolic studies were onfined to the fermentative group. These are compared serologically to the onfermentative organisms.

Methods and Materials

Strains. The following strains of PPLO were studied:

Human H-O7 and H-39, received from P. F. Smith, University of Pennsylvania, Philadelphia, Pa.

Bovine A, isolated from the prepuce of a bull by N. O. Olsen, Morgantown W.Va.

Bovine B-15 (*M. laidlawii*), isolated from a bovine genital tract by D. G. ff.

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Edward, The Wellcome Research Laboratories, Beckenham, Kent, England.

Bovine B-16, isolated from the trachea of a cow by H. E. Adler, University of California, Davis, Calif.

Caprine, strain 14, isolated from the joints of a goat by H. E. Adler.

Ovine, strain 3, isolated from a sheep with pneumonia by H. E. Adler.

Porcine (*M. hyorhinis*), isolated from the lung of a pig by W. P. Switzer, University of Iowa, Ames, Iowa.

Laidlaw B (*M. laidlawii*), isolated from sewage by P. P. Laidlaw and W. J. Elford, National Institute of Medical Research, Hampstead, England.

Avian A5969, isolated from a chicken with chronic respiratory disease by H. Van Roekel, University of Massachusetts, Amherst, Mass.

Avian S6, isolated from a turkey and received from H. E. Adler.

Avian F, isolated from a chicken and received from H. E. Adler.

Avian C, isolated from a chicken and received from H. E. Adler.

Avian 621, isolated from the trachea of a chicken by us.

Pigeon, isolated from a pigeon with coryza by H. E. Adler.

All organisms, as determined by cultural and microscopic examination, appeared to be typical PPLO.

Media. Human strains H-O7 and H-39 and bovine B-16 were grown in PPLO broth, *p*H 8.0 (Difco*) plus 1 per cent PPLO serum (Difco). The porcine strain, a very fastidious organism, was grown in fresh beef heart infusion, *p*H 8.0, containing 1 per cent PPLO serum and 1 per cent glucose. The remaining strains were grown in tryptose broth, *p*H 8.0, containing 1 per cent PPLO serum and 1 per cent glucose. No bacterial inhibitors (penicillin or thallous acetate) were added to the medium. Laidlaw B and bovine B-15 grew in basal medium without addition of serum. All other strains showed a requirement for serum. Since Laidlaw B and bovine B-15 grew much better in its presence, PPLO serum was added routinely to all media. Determinations of protein and lipid requirements were not made.

Growth conditions. Organisms were grown in 8 l. of medium in 12-l. boiling flasks at 37° C. Since preliminary experiments showed that shaking improved growth, flasks were shaken routinely on an Eberbach shaking device at 60 cpm. Inoculum for these flasks was made as follows. Stock cultures in 10 ml. of broth were inoculated into 200 ml. of broth and shaken for 24 hours. The 200-ml. culture was transferred to a 12-l. flask. Laidlaw B and bovine B-15 grew at room temperature, but grew equally well at 37° C.

For determination of end products in broth, cultures were allowed to grow for 72 hours and analyzed immediately after removal of cells by centrifugation. For manometric studies, 48-hour cultures were used.

Analytical methods. Lactic acid was determined by the method of Barker and Summerson (1941); pyruvic acid was determined by the method of Elgart and Nelson (1941).

Volatile acids were determined on a steam distillate by esterification of the acids with diazomethane (Bergmann and Segal, 1956), converted to the hydroxamic acids, and chromatographically identified by the method of Canterelli

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* Product of Difco Laboratories, Detroit, Mich.

1956). Since acetic acid was the only volatile acid formed, quantitative leterminations were made by titration of the steam distillate.

Tests for acetylmethylcarbinol (AMC), ethyl alcohol, and acetaldehyde were lone on neutral steam distillates. AMC was determined by the method of Vesterfield (1945) after oxidizing AMC to diacetyl (DA) with ferric chloride. The relative amounts of AMC and DA were not determined.

Ethyl alcohol was estimated by the microdiffusion method of Winnick (1942). Corrections were made for AMC and DA.

Acetaldehyde was determined essentially by the procedure of Huelin (1952) nd described by Jackson and Morgan (1954).

Manometric methods. Manometric studies were carried out in Warburg espirometers using conventional methods (Umbreit *et al.*, 1949). CO_2 was letermined by the direct method. In studying the dismutation of pyruvate, xygen-free N_2 was used as the gas phase. Contents of a number of flasks were ooled in order to obtain sufficient material for acetate and DA determinations.

Cell suspensions. Cells were centrifuged in a Spinco model-L centrifuge, sing the batch bowl at 30,000 g for 30 min. Cells were resuspended in M/15 hosphate buffer, *p*H 7.6. Since excessive washing decreased oxidative activity, cells were washed once in buffer and centrifuged at 32,700 g for 10 min. n a Sorval SS-1 centrifuge. Final suspensions were made in buffer to contain .0-mg. N/ml. Cellular nitrogen was determined by comparing turbidity t 420 mμ to a previously prepared standard curve relating turbidity and cellular nitrogen as determined by the micro-Kjeldahl procedure.

Oxidation of pyruvate was slow and was remedied by addition of thiamine yrophosphate (TPP), 0.01 M, and magnesium sulfate (0.01 M) to all cell uspensions before use. It was essential for best activity to use cell suspensions vithin 6 hours of harvest.

Carbohydrates employed in this study were obtained from the Difco Laboraories. Thiamine pyrophosphate (TPP), diphosphopyridine nucleotide (DPN), nd other carbon compounds were obtained from Nutritional Biochemicals Corporation.*

Serologic studies. Antigens were prepared as described above for cell uspensions. Cells were resuspended in normal saline (0.05 per cent formalin) o a density of Nephelometer 1.0.

Antiserums were prepared by intraperitoneal inoculation of 6-month-old abbits with 48-hour living broth cultures. Rabbits were given a 10-ml. noculation twice weekly for 1 month and were bled by cardiac puncture 1 veek after the last inoculation.

For tube agglutination tests, standard procedures were followed using appropriate serum dilutions. Tubes were incubated at 37° C. and read at 48 ours.

Results

Utilization of carbohydrates. Addition of 1 per cent carbohydrate to the asal medium revealed the fact that the organisms could be divided into two roups: nonfermentative and fermentative. The nonfermentative group consisted of human strains H-O7 and H-39, bovine B-16, and pigeon. The rest belonged to a fermentative group that utilized glucose, fructose, maltose, dextrin and, with some variability, glycogen, starch, sucrose, mannose, and galactose. No other carbohydrates tested were utilized. Results are shown in TABLE 1.

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* Nutritional Biochemicals Corporation, Cleveland, Ohio.

Variability of fermentation among the organisms studied appeared to be common. Avian strain A5969, when first isolated, fermented maltose and sucrose. After numerous broth passages it lost this ability and later regained it. The porcine strain, when first obtained, failed to ferment any carbohydrates. After 50 broth passages it was observed to attack glucose. Subsequent retesting revealed the additional fermentation of glycogen, dextrin, maltose, and fructose.

TABLE 1

FERMENTATION OF CARBOHYDRATES BY *MYCOPLASMA* SPP.

Carbohydrate	Human H-O7	Human H-39	Bovine A	Bovine B-15	Bovine B-16	Caprine	Ovine	Porcine	Laidlaw B	Avian A5969	Avian S6	Avian F	Avian C	Avian 621	Pigeon
Glycogen	−	−	+	+	−	+	+	+	+	−	−	−	+	−	−
Starch	−	−	+	+	−	+	+	−	+	−	−	−	+	−	−
Dextrin	−	−	+	+	−	+	+	+	+	+	+	+	+	+	−
Maltose	−	−	+	+	−	+	+	+	+	+	+	+	+	+	−
Sucrose	−	−	+	−	−	+	+	−	−	+	+	+	+	+	−
Lactose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glucose	−	−	+	+	−	+	+	+	+	+	+	+	+	+	−
Fructose	−	−	+	+	−	+	+	+	+	+	+	+	+	+	−
Mannose	−	−	+	+	−	+	+	−	+	+	+	+	+	+	−
Galactose	−	−	−	+	−	+	−	−	+	+	+	+	+	+	−
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mannitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Ribose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Xylose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Key: +, acid; −, no acid.

One per cent carbohydrate added to basal medium. Phenol red indicator. Tubes incubated 21 days at 37° C.

End products of glucose metabolism by growing organisms. Analysis of end products in broth showed that all fermentative organisms produced lactic acid, pyruvic acid, acetic acid, and AMC from glucose. Tests for ethyl alcohol and acetaldehyde were negative. Results are shown in TABLE 2.

In all cases, lactic acid was the predominant acid formed. The accumulation of pyruvic acid suggested the lack of cofactors in the medium. Jackson (1957) showed that the accumulation of pyruvate in growing cultures of *Proteus vulgaris* could be prevented by addition of DPN. However, addition of DPN to caprine and avian A5969 strains had no effect. Addition of TPP (0.01 M) and magnesium sulfate (0.01 M) were also without effect. The addition of fresh yeast extract (O'Kane, 1950) to the basal medium was tested on one avian strain (A5969). Analysis of the broth in this case revealed no detectable accumulation of pyruvate. Acetic acid was increased approximately threefold. Lactic acid concentrations remained the same. These results are similar to those reported for *Str. faecalis* by O'Kane (1950). Experiments replacing yeast extract with coenzyme A (CoA) and α-lipoic acid have not been completed. The accumulation of small amounts of acetate in all strains probably is due to the presence of low levels of the necessary cofactors in the medium.

Lactic, pyruvic, and acetic acids accounted for 90 to 100 per cent of the total acidity produced, as determined by titrating the medium to its original *p*H.

Optimum oxygen requirements for growth. Experiments showed that all fermentative strains grew under anaerobic conditions in broth and on agar plates. In no cases was growth as good as in air. Furthermore, anaerobic growth in the presence of glucose was only slightly better than growth in the basal medium alone. Subsequent studies of oxygen requirements showed that the strains grow optimally in an atmosphere from between 1 to 8 per cent oxygen and, in this respect, differed from *M. mycoides*, which was shown by Rodwell and Rodwell (1954) to grow best under high oxygen tension.

TABLE 2

END PRODUCTS OF GLUCOSE METABOLISM IN BROTH BY *MYCOPLASMA* SPP.

Organism	Lactic acid mM/l.	Pyruvic acid mM/l.	Acetic acid mM/l.	Percentage total acid recovered	AMC mM/l.
Bovine A	9.3	6.0	0.3	96.4	0.0007
Bovine B-15	5.8	5.0	0.2	90.2	0.0003
Caprine	15.1	4.8	1.2	95.9	0.0034
Ovine	15.3	5.4	1.2	99.5	0.0032
Porcine	9.6	0.8	1.0	93.4	0.0006
Laidlaw B	6.0	5.1	0.5	89.9	0.0002
Avian A5969	14.2	6.2	1.4	99.1	0.0011
Avian S6	12.4	5.1	0.5	94.4	0.0009
Avian F	10.8	4.2	1.0	96.1	0.0002
Avian C	11.0	6.3	0.4	102.3	0.0010
Avian 621	14.2	1.5	1.3	101.2	0.0005

Although further experiments were not done, it was concluded that anaerobic breakdown of glucose does not satisfy the necessary requirements for optimum growth.

Manometric studies. Of the eleven strains examined manometrically, caprine, ovine, porcine, and avian strains A5969, S6, and F showed very low endogenous respiration, $Q_{O_2}(N) = <5$. Bovine A and B-15, Laidlaw B, and avian C and 621 exhibited somewhat higher endogenous respiration, $Q_{O_2}(N) = 50$ to 70. Results presented below were corrected for this respiration. Nonfermentative strains were not studied.

Oxidation of carbon compounds by cell suspensions. The ability of cell suspensions to oxidize various carbon compounds including those of the tricarboxylic acid cycle was tested. Glucose, pyruvate, and lactate were oxidized rapidly. Acetate, α-ketoglutarate, succinate, malate, and, in some cases, oxalacetate were oxidized slowly. Citrate, acetaldehyde, and ethyl alcohol were not oxidized by any of the organisms (TABLE 3).

Crude cell extracts, prepared by rupturing cells in a Raytheon 10 kc. sonic oscillator at 4° C. for 20 min., showed no O_2 uptake with any compounds including glucose. Adding varying concentrations of methylene blue as electron carrier to the Warburg flasks also yielded negative O_2 uptakes. Cell rupture apparently destroyed the electron transport system.

Breakdown of glucose and pyruvate by cell suspensions. Quantitative manometric studies using resting cells disclosed a similar breakdown of glucose and pyruvate by the fermentative strains.

TABLE 3

OXIDATION OF CARBON COMPOUNDS BY CELL SUSPENSIONS OF *MYCOPLASMA* SPP.

Substrate	Bovine A	Bovine B-15	Caprine	Ovine	Porcine	Laidlaw B	Avian A5969	Avian S6	Avian F	Avian C	Avian 621
Glucose	76	59	261	245	101	87	293	280	245	110	143
Pyruvate	49	34	137	122	71	66	145	132	105	60	87
Lactate	53	26	148	124	62	45	192	157	129	67	101
Acetate	0	0	0	0	0	2	0	3	0	0	0
Citrate	0	0	0	0	0	0	0	0	0	0	0
α-Ketoglutarate	0	2	0	0	0	0	3	0	0	0	0
Succinate	0	7	0	0	0	0	2	0	0	0	0
Malate	0	4	0	0	0	3	7	2	0	0	0
Oxalacetate	0	9	0	0	0	5	10	3	0	0	0
Acetaldehyde	0	0	0	0	0	0	0	0	0	0	0
Ethyl alcohol	0	0	0	0	0	0	0	0	0	0	0

* All values expressed as $Q_{O_2}(N)$.
M/15 phosphate buffer, *p*H 7.6. 25 μM substrate. Incubation time 3 hours at 30° C.

Aerobically, 2.5 μM glucose and 5.0 μM pyruvate were oxidized to acetic acid and CO_2 according to the following formulas:

glucose

$$C_6H_{12}O_6 + 2\ O_2 \rightarrow 2\ CH_3COOH + 2\ CO_2 + 2\ H_2O$$

pyruvate

$$CH_3COCOOH + \tfrac{1}{2}\ O_2 \rightarrow CH_3COOH + CO_2$$

Results are shown in TABLE 4.

Anaerobically, pyruvate was dismutated to approximately equimolar amounts of lactate, acetate, and CO_2 according to the following formula:

$$2\ CH_3COCOOH + H_2O \rightarrow CH_3CHOHCOOH + CH_3COOH + CO_2$$

At the *p*H of this experiment (7.6) no AMC was detected. Results are tabulated in TABLE 5. That AMC was produced in broth and none was produced in manometric studies is perhaps explained by the fact that the optimum *p*H for AMC production is between 5 and 6 (Silverman and Werkman, 1941). In growing cultures the *p*H was lowered to the acid range, while in manometric studies it was maintained at 7.6. This may also explain the differences reported here from those of Rodwell and Rodwell (1954). These workers showed that AMC was produced anaerobically from pyruvate by cell suspensions of *M. mycoides*. The *p*H (7.4) was slightly lower than that used here.

TABLE 4
AEROBIC METABOLISM OF GLUCOSE AND PYRUVATE BY CELL SUSPENSIONS OF *MYCOPLASMA* SPP.

Organism	2.5 μM glucose*			5.0 μM pyruvate†		
	μM O_2	μM acetate	μM CO_2	μM O_2	μM acetate	μM CO_2
Bovine A	4.91	5.65	4.72	2.37	4.52	4.60
Bovine B-15	4.69	4.05	4.32	2.41	4.22	4.32
Caprine	5.09	4.93	5.18	2.59	4.91	4.90
Ovine	5.00	4.98	4.81	2.54	5.18	4.75
Porcine	4.78	5.15	4.76	2.54	5.82	4.70
Laidlaw B	4.64	5.30	4.40	2.50	4.60	5.15
Avian A5969	4.91	4.95	4.89	2.46	4.86	4.98
Avian S6	4.95	4.80	4.92	2.53	5.02	4.83
Avian F	4.78	4.73	5.09	2.43	4.82	5.05
Avian C	4.82	4.35	4.57	2.32	5.21	4.52
Avian 621	4.87	4.90	5.13	2.28	5.08	4.83
Theoretical	5.00	5.00	5.00	2.50	5.00	5.00

* $C_6H_{12}O_6 + 2 O_2 \rightarrow 2 CH_3COOH + 2 CO_2 + 2 H_2O$.
† $CH_3COCOOH + \frac{1}{2} O_2 \rightarrow CH_3COOH + CO_2$.
M/15 phosphate buffer, *p*H 7.6. 2.5 μM glucose, 5.0 μM pyruvate. Incubation time 3 hours at 30° C. Air atmosphere.

TABLE 5
ANAEROBIC METABOLISM OF PYRUVATE BY CELL SUSPENSIONS OF *MYCOPLASMA* SPP.

Organism	5.0 μM pyruvate*		
	μM lactate	μM acetate	μM CO_2
Bovine A	2.75	2.40	2.35
Bovine B-15	2.62	2.05	2.42
Caprine	2.47	2.39	2.52
Ovine	2.40	2.49	2.43
Porcine	2.52	2.61	2.48
Laidlaw B	2.30	2.35	2.22
Avian A5969	2.46	2.44	2.41
Avian S6	2.54	2.42	2.45
Avian F	2.47	2.35	2.38
Avian C	2.28	2.42	2.60
Avian 621	2.32	2.53	2.38
Theoretical	2.50	2.50	2.50

* $2 CH_3COCOOH \xrightarrow{H_2O} CH_3CHOHCOOH + CH_3COOH + CO_2$.
M/15 phosphate buffer, *p*H 7.6; 5.0 μM pyruvate. Incubation time 3 hours at 30° C. N_2 atmosphere.

The rapid oxidation of glucose and the slow O_2 uptakes demonstrated with compounds associated with terminal respiration, except with lactate and pyruvate, indicated that the major source of energy in growing cultures is obtained rom the breakdown of carbohydrate to the lactate and acetate level. Consistent with the findings of Lecce and Morton (1954) and Rodwell and Rodwell (1954), oxidation of tricarboxylic acid cycle intermediates by *Mycoplasma* spp. appeared to play a minor role, if any, in the formation of energy.

Serologic studies. Although the fermentative strains of PPLO appeared similar in the breakdown of carbohydrate, serologic examination by the tube agglutination test showed, in general, little relationship between different organisms. Human H-O7 and H-39 were serologically similar, as were caprine and ovine. Avian A5969, S6, and F cross-reacted to high titers. Avian 621 antiserum reacted weakly with A5969, S6, and F antigens. Avian 621 antigen was agglutinated only by homologous antiserum. Homologous titers ranged between 1:20 and 1:320. Results of tube agglutination tests are shown in TABLE 6.

TABLE 6

SEROLOGIC RELATIONSHIP BETWEEN STRAINS OF *MYCOPLASMA* SPP.

Antigen	Antiserum														
	Human H-O7	Human H-39	Bovine A	Bovine B-15	Bovine B-16	Caprine	Ovine	Porcine	Laidlaw B	Avian A5969	Avian S6	Avian F	Avian C	Avian 621	Pigeon
Human H-O7	40	80	0	0	0	0	0	0	0	0	0	0	0	0	0
Human H-39	80	160	0	0	0	0	0	0	0	0	0	0	0	0	0
Bovine A	0	0	80	0	0	0	0	0	0	0	0	0	0	0	0
Bovine B-15	0	0	0	40	0	0	0	0	0	0	0	0	0	0	0
Bovine B-16	0	0	0	0	80	0	0	0	0	0	0	0	0	0	0
Caprine	0	0	0	0	0	20	20	0	0	0	0	0	0	0	0
Ovine	0	0	0	0	0	40	40	0	0	0	0	0	0	0	0
Porcine	0	0	0	0	0	0	0	320	0	0	0	0	0	0	0
Laidlaw B	0	0	0	0	0	0	0	0	80	0	0	0	0	0	0
Avian A5969	0	0	0	0	0	0	0	0	0	320	80	160	0	20	0
Avian S6	0	0	0	0	0	0	0	0	0	160	160	160	0	20	0
Avian F	0	0	0	0	0	0	0	0	0	80	160	320	0	20	0
Avian C	0	0	0	0	0	0	0	0	0	0	0	0	160	0	0
Avian 621	0	0	0	0	0	0	0	0	0	0	0	0	0	160	0
Pigeon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	80

* All values expressed as reciprocal of titer.
Hyperimmune rabbit antiserum. Standard tube agglutination test.

Results of tube agglutination tests between Laidlaw B and bovine B-15 are the same as those reported by Edward (1950). Slight cross reactions between these two organisms were noted by him by means of the complement fixation test.

Discussion

Results described here showed that, basically, PPLO could be divided into two groups on the basis of carbohydrate utilization. Organisms in the fermentative group, although generally unrelated serologically, are similar in the fermentation of carbohydrates and metabolism of glucose. Glycogen, starch, dextrin, maltose, sucrose, and the hexose sugars were the only carbohydrates attacked. However, the variability of fermentation within a particular strain, s demonstrated by porcine and avian A5969, indicated that the fermentation, r lack of it, of a particular carbohydrate cannot be used as a diagnostic tool in he differentiation between strains of PPLO, especially if the isolate has been arried on media for a considerable time.

Analysis of end products of growing cultures showed these organisms to be imilar to *Str. faecalis* (O'Kane, 1950). Glucose was oxidized by this organism, ielding lactic and acetic acids and AMC. Pyruvic acid accumulated when east extract was omitted from the medium. Accumulation of pyruvic acid in rowing cultures of the one strain (A5969) tested was removed by the addition f yeast extract and not by TPP, suggesting a requirement for α-lipoic acid r CoA, or both.

Cell suspensions of PPLO supplemented with TPP and Mg^{++} oxidized yruvate rapidly to acetate and CO_2. This finding is not consistent with esults obtained by broth analysis, which showed only slight oxidation of yruvate to acetate. It is possible that the concentration of necessary cofactor, lthough very low, is sufficient to allow a complete oxidation of pyruvate to cetate when TPP and Mg^{++} are added.

Manometric studies using cell suspensions showed that the organisms studied ere similar to *M. mycoides* (Rodwell and Rodwell, 1954), which aerobically roduced acetate and CO_2 from glucose and pyruvate. Anaerobically, *M. ycoides* also dismutated pyruvate to lactate, acetate, and CO_2. Glucose as not attacked anaerobically. Anaerobic utilization of glucose by cell susensions of strains studied here has not as yet been examined. However, the act that organisms grew poorly under anaerobic conditions in the presence of lucose, and only slightly better than those without glucose, indicated that lucose was not utilized to any extent in the absence of air.

Of the 15 strains of PPLO studied, 10 serologic types were discernible.

Summary

The PPLO strains studied could be divided into two major groups based on heir ability to utilize carbohydrates.

The variability of fermentation of carbohydrates by a single strain showed hat utilization of a particular carbohydrate cannot be used as a dependable iagnostic tool in differentiating species.

Fermentative strains utilized the same group of carbohydrates. They all roduced lactic acid, pyruvic acid, acetic acid, and AMC in broth, and they ll metabolized glucose and pyruvate in a similar fashion.

Metabolically, the fermentative strains resemble *M. mycoides* and *Str. aecalis.*

Among the 15 strains, 10 serologic types could be distinguished.

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PRODUCTS OF GLUCOSE METABOLISM BY PLEUROPNEUMONIALIKE ORGANISMS*

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Introduction

The microorganisms comprising the pleuropneumonialike (PPLO) group have been assembled from diverse sources, but share certain characteristics that have been utilized to draw them together into a group. The nature of these properties has been summarized by several authors (Sabin, 1940; Edward and Freundt, 1956). The most significant of these unifying characteristics is, we believe, the lack of a cell wall. The existence of this group with its peculiar characteristics poses some fundamental biological questions; the most germane of these is the relationship of the members of this assemblage to the true bacteria.

In several respects the group is heterogeneous; it embraces organisms with a variety of properties and includes animal pathogens, commensals, and saprophytes; also, it lacks serologic unity. Another aspect of fundamental heterogeneity within the group can be based upon the ability to attack carbohydrates. One can utilize this ability to cleave the entire group into two portions. Differential attack of carbohydrates has been used extensively as an aid in the identification of PPLO (Edward, 1954; Freundt, 1954). Taxonomic use has been made of the fact that the type organism, *Mycoplasma mycoides*, as well as several other "authentic" members of the PPLO group attack carbohydrates; however, the main divisions in the classification scheme are based on source of isolation.

In 1900 Dujardin-Beaumetz showed that acetic acid was the sole volatile acid produced by cultures of *M. mycoides* in carbohydrate media. Between this early work and the modern intensive study of the type organism by Rodwell and Rodwell (1954*a*, 1954*b*, 1954*c*) there was very little work done on any of the aspects of the metabolism of the PPLO.† Rodwell and Rodwell (1954*a*) have confirmed and extended the long-standing observations of Dujardin-Beaumetz. Acetate and CO_2 are the only products of glucose oxidation by ntact or disrupted cells of *M. mycoides* (V5); furthermore, cell suspensions do not attack glucose anaerobically. We wished to determine if the pattern of attack on glucose displayed by the type organism would be shown also by the other fermentative PPLO. It was our feeling that such a study would clarify elations within the group and also to the true bacteria.

Materials and Methods

The strains chosen for our study (TABLE 1) include representatives of all he recognized members of the PPLO group that attack carbohydrates except

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† See Edward, 1954, for a review of this early work. Amino acid and oxidative metabo-sm, as well as the more recent work of Rodwell and Rodwell, are discussed elsewhere in this monograph.

M. mycoides (the importation of this organism into the United States is prohibited). Most of these strains are well known. The Goat strain is reported to be serologically related to *M. mycoides* var. *capri*, the organism of caprine pleuropneumonia (Freundt, 1957; Cordy and Adler, elsewhere in this monograph). The S6 strain is implicated in chronic respiratory disease (Adler *et al.* 1957; Adler, elsewhere in this monograph), and the Calf strain was isolated from a calf suffering from arthritis (Moulton *et al.*, 1956).

None of the strains has shown evidence of reversion to bacterial forms under routine cultivation on PPLO agar (Difco*). Similarly, only normal growth was obtained on gradient plates containing diaminopimelic acid (1 mg./ml.), or a bacterial hydrolyzate (10 per cent, v/v; prepared from *Escherichia coli*) alone and in combinations (McQuillan, 1958; Lederberg and St. Clair, 1958). Exposure to ultraviolet light has yielded no forms worthy of note here.

The only variation we have noted under ordinary conditions of cultivation is the sporadic occurrence of giant colonies (FIGURE 1) in several of the strains. Kandler and Kandler (1954) discussed the appearance of such colonies in their strains. They noted that the colonies occurred most often on the edges of plates, and attributed them to differences in nutritional requirements. In our strains similar large colonies occur randomly over the surface and appear linearly following the agar-block streak. We have attempted to isolate and propagate such colonies; however, they always yield colonies of the normal size. It should be recognized, of course, that even after single colony isolation we are probably dealing with mixed populations of clones, and that they are perpetuated by our methods of cultivation.

TABLE 1
FERMENTATIVE STRAINS OF PPLO

	Binomial name	Strain	Source	Received from
Aerobic	*M. neurolyticum*	L_5*	Mice	D. G. ff. Edward
Faculative anaerobic	*M. laidlawii*	Sewage A*	Sewage	D. G. ff. Edward
		S6	Fowl	H. E. Adler
		Calf	Calf	J. Moulton
		Goat*	Goat	H. E. Adler
	M. pulmonis	L_3* "Ash"	Mice	E. A. Freundt
Anaerobic	*M. fermentans*	G*	Human	E. A. Freundt

* Freundt, 1957.

Potentially, chemical intermediates might be accumulated in the medium if any of these strains represents a mutant. Media in which some of these strains had been grown might serve as a source of intermediates to bypass a biochemical genetic block in another strain. Experiments along these lines, crossing each strain against concentrated pools of broth from the remaining strains presently available in our laboratory, thus far have yielded only typical forms that grow in the normal manner.

It was hoped that resting cell studies using conventional manometric techniques could be utilized in these studies since they would not be subject to some of the errors inherent in growth studies carried out over a period of time in complex media. However, difficulty was encountered in obtaining satisfactory activity. Upon sedimentation, cells yielded slimy pellets that were difficult to resuspend; no activity was obtained when the cells were washed or suspended in phosphate buffers or saline. Suspensions made in fresh PPLO broth (without serum) had low activity (approximately 80 μl./hour) with or without glucose, and no significant change in glucose occurred. Presumably, the solution of this problem lies in the selection of a proper suspending medium.*

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* Product of Difco Laboratories, Detroit, Mich.

FIGURE 1. Giant colony (Calf strain) in the center of the field under the agar block, 1 nm. in diameter. Daughter colonies, following line of streaking, are of normal size even vhen well isolated (72 hours old).

Consequently, all subsequent studies on fermentation products were conlucted as growth experiments. Fermentations were carried out in unbuffered r 0.05 M K_2HPO_4 PPLO broth supplemented with approximately 1 per cent w/v) glucose (sterilized separately) and 10 per cent horse serum; this level of hosphate buffer does not inhibit growth unduly and allows a *p*H of 6 or less o be reached in 36 hours; the limiting *p*H is reached within 48 hours. Without uffering, the *p*H falls to 5 or below within 24 hours. An antifoaming agent, ntifoam,† was also added. Inhibitors were never used in any of the studies. Cultures were grown with shaking, with forced aeration using CO_2-free air, or under anaerobic conditions. Generally, cultures were allowed to reach their limiting *p*H.

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* Kandler and Kandler (1955) have discussed the effects of various suspending media on heir strains. Rodwell and Rodwell (1954*a*) routinely suspended cells of *M. mycoides* (V5) in istilled water without decreasing activity, whereas Freundt (1952) found that *M. mycoides* strain Pasteur Institute) is lysed by distilled water when it has been grown on a “glucosech medium” (446 mg. per cent).

† Product of Dow Chemical Company, Midland, Mich.

Analytical methods. Serum protein, which interferes with certain assays, was removed with trichloroacetic acid or by the Somogyi method. Conventional methods of analysis were utilized and will be outlined only briefly. Sugar was determined directly on diluted fermentation solutions by the anthrone method using glucose as a standard (Neish, 1952; Koehler, 1952). Surveys were made by paper chromatography for lactic acid (Block, 1958) and volatile acids (Kennedy and Barker, 1951; Buyske *et al.*, 1957) on concentrated ether extracts of culture solutions. To obtain volatile acids, cultures were steam-distilled, usually without deproteinization, at a *p*H of 2 to 3, until 5 volumes of distillate were collected; the distillate was titrated, concentrated, and chromatographed. Lactic acid assays were carried out on deproteinized and diluted cultures (Barker and Summerson, 1941); L(+)-lactic acid was determined enzymatically by coupling with diphosphopyridine nucleotide (Horn and Bruns, 1956).

TABLE 2
PRODUCTS OF GLUCOSE METABOLISM BY PPLO

Product	Micromole product/10 μM glucose utilized						
	Calf	S6	G*	SA	Goat	L_3	L_5
Lactic acid	19.9	19.3	15.7	10.7	6.3	5.1	4.1
Acetic acid	1.0	1.0	3.3	1.5	4.0	7.5	2.0
Micromoles sugar utilized/ml.	26.2	31.2	20.8	33.3	13.9	20.1	21.4
Percentages of carbon recovered as lactic acid	99.5	96.5	79	53.5	31	26	20
Percentages of carbon recovered	103	101.5	89.5	58	27.9	51.5	27.2

* Anaerobic conditions.

Results and Discussion

We hoped that a study comparing the pattern of products from glucose by the various fermentative strains of PPLO would contribute to our understanding of the relations of these varied forms to one another and perhaps also to known members of the Eubacteriales. Our main interest then was not to perform complete fermentation balances, but rather to establish the fermentation patterns of these strains.

The results of these studies as carried out under aerobic conditions are shown in TABLE 2. Acetic acid was the only volatile acid detected on chromatographs. Formate may be present in amounts that escaped detection. Strain S6 and Calf carry out a homolactic fermentation; *M. fermentans* may also be classed as homofermentative. The balances for the remaining strains are technically poor; however, the data show a heterofermentative pattern. Thus the fermentative strains of PPLO that have been segregated from the remainder of the group can be divided further into two groups, homofermentative and heterofermentative, on the basis of fermentation patterns. All these strains, with the exception of *M. neurolyticum*, can attack glucose anaerobically and thus differ from the type organism. It would be expected that *M. mycoides* would produce some lactate during growth, and that it would fall in the heterofermentative group, although the actual pattern it would display in cultures is uncertain. Rodwell and Rodwell (1954*a*) found that cell suspensions of *M. mycoides* (V5) produce equimolar amounts of CO_2 and acetic acid from either glucose or pyruvate under aerobic conditions; anaerobically only pyruvate is attacked, by dismutation, to yield acetate, lactate, and CO_2. Freundt (1952) mentioned that a positive Hopkins test for lactic acid is obtained in cultures (stationary, presumably) of *M. mycoides* (strain Pasteur Institute). As would be expected, the growth of the homolactic strains correlates with the rise in titratable acidity and also the fall in *p*H.

A knowledge of the nature of the isomeric forms of the lactic acid produced would be of value in further characterizing the strains. Since the acquisition of amounts of lactic acid sufficiently large to carry out the proper fractional crystallization studies would be quite difficult, we adopted an enzymatic method for L(+)-lactic acid. In some cases interfering substances were present which prevented assays; however, it appears that all the lactic acid produced by S6 and Calf is the L(+) isomer. Strains S6 and Calf thus may be characterized as being catalase-negative, nitrate-negative, homofermentative organisms producing L(+)-lactic acid.

This description is highly reminiscent of that for members of the Lactobacillaceae, particularly the streptococci. Furthermore, streptococci lack cytochromes and are also often resistant to tellurite and thallium. We elt that the possibility exists that these organisms may represent stable L orms of streptococci. Two approaches were contemplated that might estabish a relationship between these two strains and the streptococci: (1) the PPLO could be carrying bacteriophage for the streptococci which might be demonstrable if suitable hosts were provided; and (2) serologic relations might be uncovered. It is unlikely that surface antigens would be available for reaction (Sharp *et al.*, 1957). Initially, polyvalent antisera prepared against hese strains might be used against streptococci or streptococcal L forms, although a number of other experiments could be proposed; all such trials would be highly empirical.

Should it prove to be true that these strains or, for that matter, other members of the group, are stable L forms of bacteria, the question of the nature f the parental bacterial forms is raised. The diseases for which the PPLO are tiological agents are often, but not always (for example, agalactia), definitive. One wonders why similar diseases with bacterial etiological agents are not nown. The nature of the changes occurring in host-parasite interaction when bacterial form loses all or a part of its cell wall should be one of our major oncerns.

We believe then, that we have underlined an area of heterogeneity within the leuropneumonia group and have outlined a potential avenue of approach to he true bacteria.

Summary

The PPLO group is a heterogeneous assemblage that can be divided along several lines; one area of fundamental cleavage within the group can be based on the ability to attack carbohydrates. A comparative study of the end products of glucose metabolism by representatives of all the "authentic" fermentative PPLO show that these strains can be divided further into two groups, homofermentative and heterofermentative, on the basis of fermentation patterns. Furthermore, strains S6 and Calf, which are characterized as being catalase-negative, nitrate-negative, and homofermentative organisms producing L(+)-lactic acid, may represent stable L variants of members of the Lactobacillaceae. Approaches for establishing a relationship between these strains and the bacterial forms are mentioned.

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OXIDATIVE METABOLISM OF PLEUROPNEUMONIA-LIKE ORGANISMS*

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The difficulty of growing large quantities of pleuropneumonialike organisms (PPLO) for use in metabolic activities has impeded investigation in this field. The relatively few studies available indicate that the oxidative metabolism is similar for those strains isolated from human sources. The three strains investigated for their oxidative activities by Lecce and Morton[1] were found to be similar in their relatively high activity for ethanol, their lesser activity for lactate, ribose, and fructose, and their lack of activity for glucose. The low activity of these strains for carbohydrates prompted the investigation of other strains and other possible oxidizable substrates. A saprophytic strain, Laidlaw B, one bovine strain, B-15, an avian strain, J, and two strains isolated from human sources, O7 and Campo L, were used in this study. The organisms were grown in large-volume broth cultures (Morton *et al.*)[2] enriched with 1 per cent Difco Bacto-PPLO serum fraction (Smith and Morton),[3] harvested by centrifugation in a Sharples supercentrifuge, and washed several times with sterile physiological saline. The saprophytic strain grew better when the level of serum fraction was reduced below 1 per cent. A Warburg respirometer was used to follow the oxidation of the various substrates.[4] The conditions of each experiment were as indicated. Sonication indicates an exposure of saline-washed cells to vibrations in a 9 kc. Raytheon magnetostrictor vibrator for a period of 20 min. The sonicate was not centrifuged before use in these experiments. The data presented are based on many repeated experiments and are characteristic of the results occurring most frequently. Some variation in activity is undoubtedly due to the limited growth of some strains in the basal medium.

TABLE 1 shows the range of oxidative activity between the various strains of PPLO for the substrates glucose, sodium acetate, and ethanol. The human strains could be characterized by the relatively slight amount of oxygen taken up in the presence of acetate and the lack of activity with glucose. The activity of the avian strain for glucose was higher than that shown by the bovine or saprophytic strain. These three strains displayed almost identical activity in the presence of sodium acetate.

Several of the short-chained fatty acids were examined for the ability to serve as oxidizable substrates for these strains of PPLO. The results of several such experiments may be seen in TABLE 2. There does not appear to be a significant difference among the various strains, with the exception of the saprophytic strain, in the ability to oxidize the sodium salts of propionate and butyrate. The addition of coenzyme A (CoA) at the level of 1 mg. per reaction vessel did not result in an increased activity directed against the fatty

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acid. On the contrary, in most instances there was a drop in activity. This may be the result of the increased endogenous activity of the whole cells in the presence of CoA.

In the preceding experiments where fatty acids were utilized as substrates for measuring oxidative activity, it was noted that a 1- to 2-hour lag period occurred before the rate of oxygen uptake increased more rapidly than did the endogenous activity. In subsequent experiments various cofactors were added to the reaction mixtures in attempts to lessen this lag period.

TABLE 1
OXIDATIVE ACTIVITY BY CERTAIN STRAINS OF PPLO

Strains	Oxygen uptake (μM)			Reaction time (hours)
	Substrate			
	Ethanol	Glucose	Acetate	
Laidlaw B	4.3	3.7	7.6	4
J	—	14.7	7.9	4
B-15	—	6.7	9.7	4
O7	—	0	1.5	5
Campo L	2.6	—	0.6	2

Each Warburg vessel contained washed whole cells suspended in physiological saline at a concentration of 1 mg. cellular nitrogen per ml.; 100 μM substrate; and M/15 phosphate buffer, *p*H 7.5. Final volume of 3 ml.

TABLE 2
FATTY ACID OXIDATION BY CERTAIN STRAINS OF PPLO (WHOLE CELLS)

Substrate	Oxygen uptake (μM)				
	Strain				
	B-15	Campo L	J	O7	Laidlaw B
Acetate + CoA	9.7	4.8	7.9	5.7	1.4*
Propionate + CoA	0.1	2.6	—	0	0
Butyrate + CoA	0	1.9	—	2.2	0
Propionate	3.9	3.4	3.8	1.5	0
Butyrate	7.0	4.0	7.5	6.9	0

* Included 10 μM ATP in reaction mixture.

Reaction vessels contained washed whole cells at a concentration of 1 mg. cellular N/ml. of physiological saline; 100 μM sodium salt of the fatty acids; 1 mg. CoA; M/15 phosphate buffer, *p*H 7.5. Final volume, 3 ml. Reaction time was 4 hours.

The activity of the whole cell versus lysed cells of the human strain O7 is shown in TABLE 3. The cells were lysed by sonication, and the sonicate was not centrifuged before testing. Under these experimental conditions the activity of the lysed cells was from one third to two thirds that of the whole cells. The addition of 1 mg. of diphosphopyridine nucleotide (DPN) to the reaction vessel containing the lysed cells did not increase the final activity or lessen the lag period.

With the fatty acids, acetate, propionate, and butyrate as substrates, the amount of carbon dioxide released was equivalent to the amount of oxygen taken up during the reaction, with the exception of butyrate oxidation by whole cells of strain Campo L (TABLE 4). The addition of 100 μM of acetate and 1 mg. of CoA to propionate and butyrate did not increase the final oxidation activity and had no effect on the lag period. Data are presented in TABLE 5 pertaining to the oxidation of other short-chained saturated fatty acids. With some of the fatty acids a comparison was made using no preincubation of the cells with a preincubation period of one hour. The total reaction time, 3 hours, during which the fatty acid substrate was present in the reaction mixture was the same in all instances. It can be noted in all experiments listed that the preincubation resulted in a higher final value. However, the lag period was not altered appreciably. In the instance of laurate it was noted that the addition of this fatty acid resulted in a depression of the endogenous rate of respiration by these cells.

TABLE 3
FATTY ACID OXIDATION BY PPLO STRAIN O7 (WHOLE VERSUS LYSED CELLS)

Substrate	Oxygen uptake (μM)		
	Whole cells	Lysed cells	Lysed cells + DPN
Acetate	1.5	0.7	0.3
Propionate	0	0	0.2
Butyrate	0.6	0.5	0.3
Butyrate*	2.9	0.9	0.7
Butyrate†	10.6	6.6	—
Caprylate†	1.7	0.1	—

* Different lot of cells, preincubation period was 30 min.; reaction time, 3 hours.
† Third lot of cells, preincubation period was 1 hour; reaction time, 4 hours.
Warburg vessels contained 1 mg. cellular N in 1 ml. saline; M/15 phosphate buffer, *p*H 7.4; fatty acids, 20 μM; 1 mg. DPN. Total volume was 3 ml. Preincubated mixture without fatty acid for 1 hour. Reaction time, 4 hours.

TABLE 4
FATTY ACID OXIDATION BY WHOLE CELLS OF PPLO STRAINS O7 AND CAMPO L

Substrate	Oxygen uptake (μM)		Carbon dioxide released (μM)	
	O7	Campo L	O7	Campo L
Propionate	1.5	3.4	1.0	2.5
Butyrate	4.6	4.0	3.2	1.5
Acetate + CoA	5.7	4.8	6.2	5.0
Acetate + CoA + propionate	2.0	2.6	2.6	2.8
Acetate + CoA + butyrate	2.2	1.9	1.3	1.9

Vessels contained fatty acid, 100 μM; CoA, 1 mg.; 1 mg. cellular N in 1 ml. physiological saline; M/15 phosphate buffer, *p*H 7.5. Total volume, 3 ml. Reaction mixture incubated for 1 hour before adding the substrate. Incubated 4 hours after the addition of substrate.

The exchange of oxygen and carbon dioxide by an avian strain of PPLO, strain J, in the presence of acetate, propionate, and butyrate is shown in TABLE 6. With these substrates the amount of oxygen taken up is nearly equal to that of the carbon dioxide evolved. In the experiment in which this strain was examined for the ability to oxidize succinate, the substrate depressed the endogenous respiration.

Additional experiments using such additives as tri(hydroxymethyl)aminomethane buffer (Tris), *p*H 7.4; CoA, 1 mg.; ATP, 10 μM; AMP, 10 μM; DPN, 1 mg.; TPN, 1 mg.; flavin adenine dinucleotide at a final concentration of 10^{-4}; $MgCl_2$, 5 μM; acetyl phosphate as its lithium salt, 3 μM; and Bacto-PPLO serum fraction at a 1 per cent level did not have an appreciable effect either on the lag period or on the final extent of oxidation of the fatty acids butyrate and caprylate. The sterols, cholestane and cholestan-3-one, seemed to inhibit oxidation of butyrate by whole cells of strain O7, but this was found to be due to the organic solvent used for dissolving these sterols. It was found that low levels of cholesterol and lecithin stimulate endogenous oxygen

TABLE 5

FATTY ACID OXIDATION BY PPLO STRAIN O7 (WHOLE CELLS)

Substrate	Preincubation time (hours)	Oxygen uptake (μM)
Acetate	0	0
	1	1.5
Propionate	0	0
	1	1.5
Butyrate	0	2.4
	1	10.6
Caproate	0	0.8
	1	1.0
Caprylate	1	9.6
Isobutyrate	½	1.5
Valerate	½	4.5
Isovalerate	½	3.9
n-Heptanoate	½	3.0
Nonanoate	½	1.1
Decanoate	½	2.9
Laurate	1	0*

* Less than the endogenous.

Vessels contained 1 mg. cellular N in 1 ml. physiological saline; M/15 phosphate buffer, *p*H 7.4; fatty acids, 20 μM; total volume, 3 ml. Reaction time, 3 hours.

TABLE 6

FATTY ACID OXIDATION BY AN AVIAN STRAIN PPLO (J)

Substrate	(μM)	
	Oxygen uptake	Carbon dioxide released
Propionate	3.8	1.6
Butyrate	7.5	6.6
Acetate	7.9	9.7
Acetate—CoA	7.9	9.6
Succinate	0*	0*

* Less than endogenous values.

Vessels contained 1 mg. cellular N in 1 ml. saline; M/15 phosphate buffer, *p*H 7.5; fatty acids, 100 μM; succinate, 500 μM; CoA, 1 mg. Total volume, 3 ml. Reaction time was 4 hours.

uptake by washed whole cells of strain O7 PPLO. However, in the presence of an oxidizable substrate, such as butyrate, these substrates inhibit oxidative respiration. The lag phase noticeable in the studies with fatty acid oxidation by whole and lysed cells suggests the necessity for complete utilization of an endogenous substrate prior to attack of the externally supplied substrate.

Summary

Metabolic studies utilizing a Warburg respirometer show that certain strains of PPLO oxidize substrates such as ethanol and short-chained saturated fatty acids. The activity of lysed cells is approximately one third to two thirds that of whole cells. Strains of these organisms isolated from human sources do not appear to attack glucose, but do show relatively good activity against butyrate, caprylate, valerate, and certain other short-chain fatty acids. The saprophytic strain examined could not utilize butyrate or propionate as a substrate for oxidative respiration under the conditions of these experiments. A 1- to 2-hour lag period was apparent after contact of the whole or lysed cells and the fatty acid substrate before a maximum rate of activity was detectable. No procedures were found that could shorten this lag period.

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AMINO ACID METABOLISM OF PPLO*

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An examination of the amino acid metabolism of pleuropneumonialike organisms (PPLO) of human origin was prompted by the negative results obtained by Lecce and Morton[1] for reductive activity toward substrates of carbohydrate nature. Thus, it was noted that of most of the Emden-Meyerhof and Krebs intermediates were inactive, yet significant activity was noted with lactate and the monohydric alcohols. Because of the fact that media capable of supporting optimal growth contained no appreciable quantities of monohydric alcohols and because of the capacity of PPLO to oxidize these alcohols, it was felt desirable to examine the possibility that amino acids might be the carbon and energy source for these strains. Since strains of avian origin and saprophytic strains are capable of oxidizing carbohydrates, the study was limited to those organisms obtained from humans.

Nutritional Studies

The initial efforts to determine whether amino acids could serve as the major carbon source were directed toward a qualitative estimation of growth obtained in a chemically defined medium.[2] The development of a synthetic medium capable of supporting growth, although suboptimal, favored the foregoing assumption, since the only compounds present in substrate amounts were glutamine, glutamic acid, arginine, aspartic acid, and possibly phenylalanine. The implications from these results were that it was advisable to examine the catabolic activity toward these particular required amino acids. Actually, it appeared that these strains of PPLO possessed an extensive synthetic, but a poor catabolic, capability.

In view of the possibility that a nonrequired amino acid might also serve as a carbon source, the next approach constituted an examination of the utilization of amino acids by resting cells.[3] The experiments were run under both aerobic and anaerobic environments. Utilization of a given amino acid was measured by assaying its loss by chromatographic procedures. As shown in TABLE 1, of the 18 amino acids and glutamine employed, utilization of 9 occurred in 3 hours. Tyrosine, tryptophan, and aspartic acid disappearance occurred mainly under anaerobic conditions, while loss of histidine, leucine, and threonine occurred only in an aerobic atmosphere. The disappearance of arginine and glutamine was complemented by the appearance of citrulline and glutamic acid, respectively. However, in neither case was quantitative recovery of the substrates in the form of these 2 reaction products achieved. The results in TABLE 1 indicate that glutamic acid is also utilized. On the other hand, when citrulline, a reaction product from arginine, was incubated with resting cells, no utilization occurred. As will be noted later, this discrepancy was found to

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be due to the impermeability of the cell to citrulline. The disappearance of glutamic acid resulted in the appearance of a Ninhydrin-negative but isatin-positive area on paper chromatographs.

Measurement of the loss of amino acids when incubated with resting cells was supplemented with determinations of ammonia release by both whole and sonicated cells at alkaline and acid *p*H. Ammonia evolution at alkaline *p*H paralleled the results of utilization of arginine, glutamine, and aspartic acid shown in TABLE 1. Deamination was also apparent at *p*H 6, as shown in TABLE 2. It may be noted that, although no difference occurred between the amount of ammonia evolved by whole and broken cells from arginine, ammonia release from citrulline took place only with lysed cells. Glutamine was not included in this table since its degradation is complicated by two reactions, which will be discussed later. Essentially, only three compounds, glutamine, arginine, and citrulline, were actively deaminated.

TABLE 1

UTILIZATION OF AMINO ACIDS BY RESTING CELLS OF STRAIN 39

Amino acid	Gaseous environment	Amino acid utilized (μM)
L-Arginine	Aerobic	42.0
	Anaerobic	42.0
L-Glutamine	Aerobic	38.8
	Anaerobic	38.8
L-Glutamic acid	Aerobic	38.3
	Anaerobic	20.2
L-Aspartic acid	Aerobic	5.0
	Anaerobic	17.5
L-Threonine	Aerobic	13.7
	Anaerobic	0
L-Leucine	Aerobic	10.3
	Anaerobic	0
L-Histidine	Aerobic	11.3
	Anaerobic	0
L-Tyrosine	Aerobic	1.9
	Anaerobic	10.5
DL-Tryptophan	Aerobic	5.3
	Anaerobic	13.9

TABLE 2

DEAMINATION OF AMINO ACIDS AT *P*H 6

Amino acid	Whole cells	Lysed cells
	μM NH_3/mg. N in 3 hours	
Arginine	19.0	19.1
Aspartic acid	0.6	0
Citrulline	0	3.0
Phenylalanine	1.6	0
Threonine	1.0	0
Tyrosine	1.8	0

The ability of PPLO of human origin to decarboxylate amino acids was studied at acid and alkaline *p*H with whole and lysed cells. No CO_2 evolution was noted with any amino acid with the exception of citrulline. As in the case of deamination, CO_2 evolution from citrulline was observed only with lysed cells.

Since such few amino acids were degraded when present as the sole substrate, it was of interest to examine the possibility of a Stickland-type reaction that is common among many anaerobic bacteria. Incubation of whole cells with alanine or valine as the H^+ donors and glycine or proline as the H^+ acceptors resulted in completely negative results. No O_2 uptake, ammonia evolution, or amino acid loss occurred during the reaction period of two hours. Observations of the results to this point were indicative of the dubious role of amino acids as the carbon and energy source. The only optimistic result was that, in contrast to their activity toward carbohydrates, these strains of PPLO did exhibit some meager enzymatic activity toward a few amino acids.

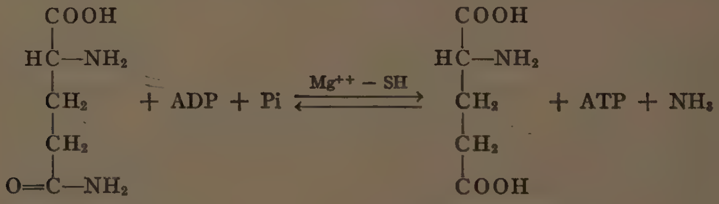
Any study of amino acid metabolism is incomplete without the inclusion of transamination. This phase of my study on the general activity of PPLO toward amino acids was conducted prior to the development of a synthetic medium. Following the usual procedure, I selected the then available amino acceptors, pyruvate, oxalacetate, and α-ketoglutarate. This proved to be a frustrating selection, as will be seen. Needless to say, I obtained no transamination between these acceptors and a wide variety of amino donors, including glutamine and asparagine. The exception was pyruvate, from which some alanine was detected after incubation with glutamine. The negative results received an explanation following the nutritional study of amino acid requirements. Both aspartic and glutamic acids, the compounds formed when oxalacetate and α-ketoglutarate are aminated, were found to be absolute requirements. Since so few amino acids are nutritional requirements, a more cautious selection of α-keto analogues undoubtedly would result in the demonstration of transaminase activity.

Although only a few amino acids were shown to be degraded, it was thought possible that these particular compounds might play an important role as suppliers of energy. Therefore, a more detailed examination of the metabolism of glutamine, glutamic acid, aspartic acid, arginine, citrulline, and ornithine was undertaken.

Glutamine

Among the amino compounds acted upon by PPLO, glutamine is by far the most reactive. Aside from its probable participation in transamination reactions as indicated by its ability to serve as amino donor in alanine transamination, two other reactions were detected and studied.[4] Hydrolytic deamidation occurs at alkaline *p*H, the optimum being *p*H 8.0. The reaction products are glutamic acid and ammonia and are formed in equimolar amounts comparable to the loss of glutamine. No cofactor requirement was demonstrated. The reaction is specific for glutamine with asparagine exhibiting no activity. Inhibitors such as iodoacetate, arsenite, cyanide, fluoride, and semicarbazide at 10^{-2} M levels were ineffective. The reaction was not affected by the presence of large amounts of glutamic acid. Only Bromsulphalein, a compound inhibitory for mammalian and bacterial glutaminases, was inhibitory.

The reaction involving glutamine that appears important in regard to energy release is phosphorolytic deamidation. It possesses a *p*H optimum around 6.0. Inorganic phosphate, Mg^{++}, and adenosine diphosphate (ADP) are absolute cofactor requirements. For each mole of glutamine, inorganic phosphate, and ADP disappearance there was equimolar formation of glutamic acid, ammonia, and adenosine triphosphate (ATP). The reaction scheme is shown in FIGURE 1. Further evidence of the phosphorolytic nature of the reaction was obtained by the demonstration of typical arsenolysis of glutamine, when arsenate was substituted for phosphate. The arsenolysis reaction was sensitive to fluoride. Similar to hydrolytic deamidation, this reaction was specific for glutamine. As may be noted in the reaction scheme, the reaction is reversible. Incubation of cell extracts with glutamic acid, ATP, ammonium chloride, Mg^{++}, and cysteine resulted in the formation of glutamine. Since the reaction is reversible, it was of primary importance, in determining its contribution to the energy supply of the cells, to establish the equilibrium values of the forward and reverse reactions. The degradation reaction, that is, ATP formation, was found to be favored, the equilibrium value being 4. The synthetic reaction, that is, glutamine formation, had a value of 2×10^{-2}.



Glutamine

Glutamic acid

FIGURE 1. Reaction scheme for phosphorolysis of glutamine.

This finding favorable to the argument that glutamine does serve as an energy source was supported further by determination of inorganic phosphate uptake in the presence of a reaction utilizing the ATP formed, for example, hexokinase + glucose. In the presence of 1.0 μM of ATP, 14.9 μM of inorganic phosphate was esterified as glucose-6-phosphate. Thus, the forward reaction shown in FIGURE 1 probably does contribute to the energy requirements of these PPLO.

Glutamic Acid

Glutamic acid, whether resulting from the described reactions of glutamine or added as a substrate, disappears without the production of ammonia or α-ketoglutarate. No ammonia or CO_2 evolution occurred under any of many varied conditions. This disappearance of glutamic acid was concomitant with the appearance of increasing levels of an isatin-positive spot on paper chromatographs. The blue isatin-reaction-product complex indicated the production of a cyclic compound. Comparative chromatography showed that the unknown and Δ^1-pyrroline-5-carboxylic acid possessed identical R_f values and were additive when chromatographed together in three different solvents.[5] Proline, hydroxyproline, and pyrrolidone-5-carboxylic acid possessed R_f values distinct from the unknown. Spectrophotometric absorption curves of the isatin complexes of the unknown and Δ¹-pyrroline-5-carboxylic acid were identical, giving peaks at 565 and 625 mμ. It was concluded that the unknown was Δ¹-pyrroline-5-carboxylic acid, a compound shown by Vogel and Davis[6] to be in nonenzymatic equilibrium with glutamic-γ-semialdehyde produced by glutamic acid reduction in *Neurospora*. The cofactor requirements were shown to be Mg^{++} and some high-energy phosphate donor such as ATP or ADP. The optimum *p*H of the reaction was 7.5, although there was no sharp definable peak. The conversion of glutamic acid to the pyrroline carboxylate was inhibited by compounds interfering with phosphorylation, such as arsenite, and by semicarbazide, which could inactivate an aldehyde intermediate by complex formation. The pyrroline carboxylate, added as the synthetic compound or

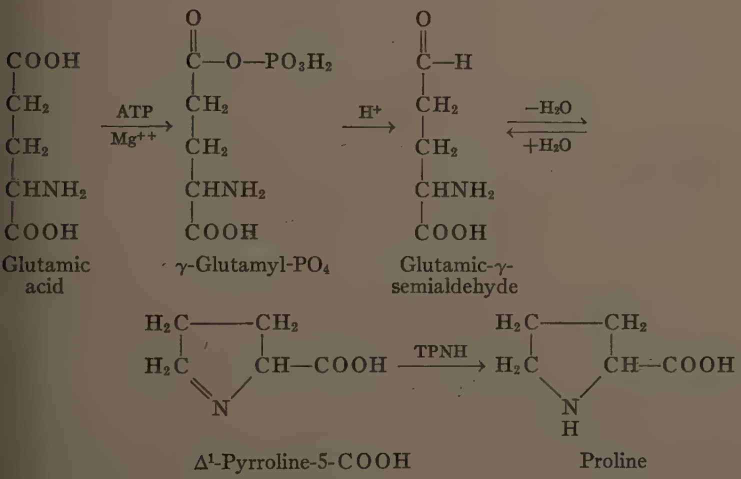


FIGURE 2. Reaction scheme for conversion of glutamic acid to proline.

as that produced from glutamate, was shown to be reduced by these PPLO through the specific action of reduced triphosphopyridine nucleotide (TPNH) to yield proline. DPNH was inactive. The proline formed was identified chromatographically. The over-all reaction scheme of glutamic acid to proline is shown in FIGURE 2. The γ-glutamyl phosphate intermediate is postulated, whereas the other intermediates are known. It is unlikely that γ-glutamyl phosphate occurs as an enzyme-free intermediate since no hydroxamic-reactive compound could be detected. It is also unlikely that the over-all reaction is reversible, since proline and the pyrroline carboxylate fail to replace he glutamate requirement for growth. The fact that glutamic acid is not deaminated or decarboxylated and does not participate in transamination eactions, but appears to be involved only in the synthesis of glutamine and proline, prompts the conclusion that the function of glutamic acid in these strains of PPLO is solely anabolic.

Aspartic Acid

A detailed examination of aspartic acid metabolism by these PPLO has not been accomplished. Nevertheless, some fundamental facts concerning its metabolism have been obtained; from these it is possible to establish or predict its probable functions. Some of the data presented here were obtained at the University of Pennsylvania by N. Somerson.* As shown previously, aspartic acid disappears when incubated with intact resting cells. Less than one half of this disappearance can be accounted for by deamination. Thus, approximately 17 μM of aspartic acid is lost, but only a maximum of 4 μM of ammonia can be detected. This result is indicative of more than one reaction. In his experiments on deamination of aspartic acid Somerson demonstrated an apparent requirement of some cofactor contained in the culture supernatant or in the culture medium. The *p*H optimum for deamination appeared to be alkaline to *p*H 7.0. Because deamination accounts for less than 25 per cent of the aspartic acid utilized and because these cells possess a system for synthesis of proline from glutamic acid, it is not unreasonable to predict that they also possess the analogous system for the synthesis of homoserine and threonine from aspartic acid. Neither of the former two compounds is a growth requirement. The mechanism shown by Black and Wright[7] for bakers' yeast first results in the production of β-aspartyl phosphate through the participation of ATP, followed by its reduction by TPNH to aspartic-β-semialdehyde, and further reduction by DPNH or TPNH to homoserine. The absolute growth requirement for aspartic acid belies a need for anabolic rather than catabolic activity.

Arginine

Next to glutamine, arginine is degraded most readily by these human strains of PPLO. Arginine disappearance is concomitant with citrulline appearance. For each mole of arginine lost, a mole of ammonia is formed. Even though quantitative recovery of arginine as citrulline is impossible without preventing the further degradation of citrulline, quantitative conversion to citrulline is the sole mechanism of arginine catabolism in these strains. In no instance was direct production of ornithine from arginine by an "arginine dihydrolase" type reaction noted. Although arginine may function in other reactions, these are undoubtedly anabolic in nature. The desimidation of arginine to citrulline is of unknown significance unless it serves as a supply of citrulline for an energy source. As will be noted later, this is unlikely. The desimidation reaction itself is hydrolytic and does not serve to supply energy.

Citrulline

Citrulline offered another of the gradually decreasing possibilities of any energy source. Like glutamine, citrulline can undergo a phosphorolytic cleavage to yield a high-energy phosphate.[8] Ornithine was shown to be a reaction product, and the reaction proceeded anaerobically as well as aerobically. Cofactor requirements were found to be inorganic phosphate and a phosphate acceptor. ADP is a slightly better phosphate acceptor than adenosine monophosphate. No metal requirement could be established. Added Mg^{++} was inhibitory, but it is probable that enough bound metal was present to allow the reaction to proceed.

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The *p*H optimum for citrulline conversion to ornithine was 5.5. The reaction was shown to behave as a typical phosphorolysis toward inhibitors of phosphorylation. Stoichiometric analysis revealed the fact that, for each mole of citrulline degraded, one mole of inorganic phosphate disappeared, and one mole each of ornithine, ammonia, CO_2, and ATP was formed. The equilibrium value of this reaction was approximately 5×10^{-1}. This value, together with the fact that under optimal conditions no more than 5 μM of citrulline were degraded, raised doubt that the reaction was of significant importance in energy release. Even coupling the reaction with hexokinase and glucose did not allow greater total activity. An explanation for this weak over-all activity was forthcoming when ornithine was incorporated into the reaction mixture. Ornithine, at a level of 12 μM, almost completely abolished the reaction.

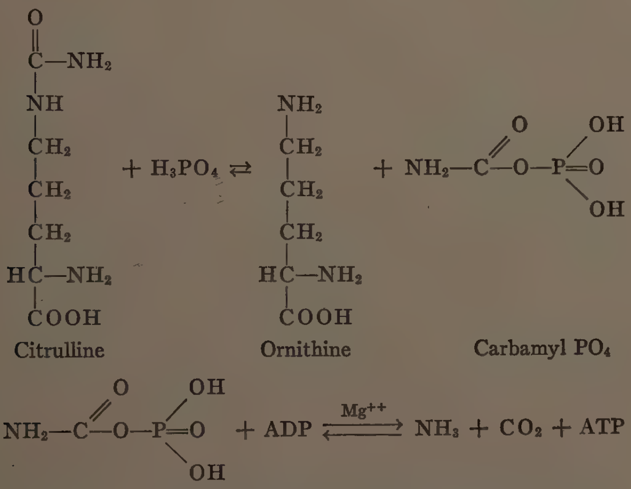
Citrulline
Ornithine
Carbamyl PO_4
$+ H_3PO_4 \rightleftarrows$
$+ ADP \xrightleftharpoons{Mg^{++}} NH_3 + CO_2 + ATP$

FIGURE 3. Reaction scheme for phosphorolysis of citrulline.

Accumulation of a phosphorylated intermediate would be indicative of an equilibrium favoring citrulline degradation. No such intermediate could be detected with hydroxylamine, although this compound was inhibitory for the reaction. Analysis of the rate of change of the reaction components indicated no such accumulation. Jones *et al.*[9] had shown that carbamyl phosphate was intermediate in the synthesis of citrulline from ornithine. Although this compound could not be detected during citrulline degradation because of its lability and reactivity, incubation of the synthetic compound with ornithine resulted in rapid citrulline synthesis. In the absence of ornithine and in the presence of ADP, carbamyl phosphate was rapidly degraded to CO_2, ammonia, and ATP. The equilibrium of the reaction synthesizing citrulline was about 5×10^2 or one thousandfold greater than citrulline degradation. The over-all reaction scheme is shown in FIGURE 3. These results indicate that, unless ornithine is utilized as rapidly as it is formed from citrulline, the synthesis of citrulline is a far more favored reaction.

Ornithine

A detailed study of ornithine metabolism has not been accomplished. It has been found that under alkaline conditions, that is, *p*H 8, ornithine is lost. This loss is attended by the appearance of an isatin-positive compound. It is conceivable that δ-transamination or deamination could give rise to glutamic-γ-semialdehyde and thus provide an intermediate for proline synthesis. Any other pathway of degradation is a matter of pure speculation at this time. Thus far, data fail to answer the question whether ornithine is removed rapidly enough to force citrulline degradation.

Conclusions

Initially, the answer sought was whether amino acids serve as the sole energy and carbon source for the human strains of PPLO under study. This answer must be a definite "No." The requirement of a rather meager number of amino acids for growth indicates an extensive synthetic capability. Of the amino acids that are degraded, all appear to lead to some synthetic reaction rather than to complete oxidation. Thus, glutamine yields glutamic acid, which enters into proline synthesis. Aspartic acid loss does not yield much ammonia, nor is there O_2 uptake. Arginine yields citrulline which, under favorable circumstances, is converted to ornithine which, in turn, enters into synthetic reactions. Citrulline, if it were permeable, could give rise to arginine. On the other hand, two compounds, glutamine and citrulline, can undergo phosphorolytic cleavage to yield high-energy phosphate. However, the end products are used for synthesis and necessarily limit the capacity of the cell to break down amounts of these compounds sufficient to yield all the energy required for growth and metabolism.

Although it is inconceivable that amino acids could give rise to compounds more readily oxidized, the results of this study indicate that amino acid metabolism of these strains of PPLO is predominantly anabolic in nature.

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DISCUSSION OF PART IV

PAUL F. SMITH (*University of Pennsylvania, Philadelphia, Pa.*), Has anyone ever determined whether L forms contain sterol, whether all L forms require sterol for growth, and whether the need of sterol is the basis for the serum requirement for growth of L forms?

MARY MEDILL-BROWN (*University of Pennsylvania, Philadelphia, Pa.*): The penicillin-induced L forms of *Proteus* with which I have worked grow very well in a completely synthetic medium containing a mixture of amino acids, phosphate buffer, glucose, and nicotinamide. This is also the basic synthetic medium for the rod forms. The L forms differ from the rod forms in that the addition of such substances as yeast extract or peptone to the basic synthetic medium inhibits growth of the L forms, but not of the rods. It stimulates the growth of rods over that obtained in the synthetic medium alone. Difco PPLO serum fraction added to the crude medium stimulates the growth of the L forms whereas, when added to the synthetic medium, it inhibits the growth of the L forms. The same results are obtained with ascitic fluid and horse serum.

I compared ether-soluble lipids of both rods and L forms, the reducing sugar after hydrolyzing both the rods and the L forms, and the total phosphate and acid-soluble phosphate; in all experiments there was no difference between rods and L-forms. This perhaps is a partial answer to your question.

SMITH: Will Rodwell comment, particularly in regard to the requirement for oleic acid or Tween 80 by bovine PPLO?

A. W. RODWELL (*Animal Health Research Laboratory, Victoria, Australia*): I have not assessed finally the Tween 80 requirement. I was postponing that until I had satisfied all the other growth requirements, when I should be in a better position to assess it. I think it is quite possible that it is not a specific fatty acid requirement. It may be simply a surface-depressant activity, as you suggest for the phospholipid. I have not shown any requirement for phospholipid by *Mycoplasma mycoides*. The serum fraction, Fraction C, as originally prepared, did contain approximately 4 per cent of phospholipid, but that was not essential because, after its removal by ethanol-ether extraction, the residue was still active. However, when employing the extracted protein, the optimal cholesterol concentration was reduced by one fifth, that is, from about 0.05 to 0.01 M per ml.

I wonder if the cholesterol found by Smith is firmly bound or is in chemical combination in PPLO? Westphal has shown that isolated *Escherichia coli* lipopolysaccharide binds both sterol and protein, and binds sterol very firmly. I think the *E. coli* lipopolysaccharide binds about 100 parts of cholesterol so firmly that it cannot be removed by solvent extraction. We do not know whether the galactan of *M. mycoides* binds cholesterol, but I wonder whether the cholesterol found by Lynn was just bound on some structure or was actually in chemical combination.

SMITH: We know that the cholesterol of PPLO is extractable with alcohol-ether. The only other finding that might bear light on this is the result of preliminary work with C^{14}-cholesterol. The radioactive cholesterol is taken up, approximately 60 to 75 per cent being located in the residue fraction.

When these resting cells are incubated in the presence of soluble nonradioactive sterol, there is no exchange of sterol between the supernatant medium and these resting cells. This might indicate that the sterol is firmly bound, but probably not in chemical combination.

Emmy Klieneberger-Nobel (*The Lister Institute of Preventive Medicine, London, England*): I have been interested particularly in the work of Brown and Rodwell. I feel that the approach of Smith will help us solve those problems of distinction between the various groups of organisms. This is a much more direct approach than, for example, the electron microscopical one with which there are difficulties of interpretation in solving the problem of minute development and methods of reproduction. Of course, these problems also must be solved but, at the moment, I feel that this is an excellent approach that gives us new information.

Smith: A paper published by Klieneberger-Nobel and a co-worker several years ago described the association of cholesterol with these organisms.

Harold Neimark (*University of California, Los Angeles, Calif.*): I believe that we should bear in mind that there may be a spectrum of various cell-wall mutants, should these organisms prove to be a group of L forms. This may help explain why there are differences in osmotic susceptibility and permeability. This, of course, is extremely speculative at present.

Comment: I do not understand the difference cited by Medill-Brown in her preparation of protoplasts from *Proteus* and from L forms.

Medill-Brown: The protoplasts were prepared by adding penicillin to an actively growing rod culture, that is, log-phase cells. In the case illustrated, the penicillin was added to the growth flask after 6 hours of growth, followed by agitation on a shaker. After the addition of penicillin, about 100 per cent of the cells become spherical. These are what I called protoplasts.

The L forms are prepared in two ways. One preparation makes use of the L-type colony growing on agar. The other preparation was derived as follows: an agar block containing colonies is transferred to the same broth medium. This culture is agitated on a shaker, and at 48 hours a transfer giving a 1:100 dilution and leaving the agar block behind is made into fresh broth medium containing 400 U. penicillin/ml. This culture is agitated on a shaker for 48 hours.

Question: Where do the L forms originate?

Medill-Brown: The L forms can be derived both from protoplasts and from rods. The ones that I described were derived by merely plating *Proteus* rods on an agar plate containing penicillin.

Klieneberger-Nobel: Are the forms you call motile L forms really stable L forms?

Medill-Brown: No. They are penicillin-induced.

Klieneberger-Nobel: I think it is better to designate only stable L forms as L forms, and call the others transformation forms. I am convinced that stable L forms never are motile.

Julius Fabricant (*Cornell University, Ithaca, N.Y.*): I suggest to any group interested in the chemistry of these organisms that a very fertile field has been neglected, that is, the relationship of pathogenicity and nonpathogenicity in these different strains to their biochemical reactions. There is a good tool available for those interested in this problem in the poultry strains. We have pathogenic and nonpathogenic strains, both of which are capable of growing well, and a cheap experimental animal. There is an opportunity to learn how to grow pathogenic strains readily and compare their biochemical mechanisms. This has, I think, a wider application than just their relationship to poultry disease. I am sure there are many strains in other animals that have not been isolated, simply because we have not yet learned how to grow these types of strains in media.

SMITH: We do not know whether any human strains are pathogenic. We have some that are very different physiologically, that is, some are very poor growers and nutritionally different from others. We have always contended that we should begin with the simple things first, in order to have some basis from which to make comparisons. I agree that Fabricant's suggestion can be applied to the avian strains.

HARRY E. MORTON (*University of Pennsylvania, Philadelphia, Pa.*): Fabricant's suggestion that the metabolism of virulent and nonvirulent strains of PPLO should be studied is very good. The following precaution should be noted. Much work has been done on the metabolism of pathogenic and nonpathogenic strains of organisms that have been grown *in vitro*. A large volume of literature has developed on the properties of pathogenic organisms grown in an artificial environment. Pathogens should be grown in the animal body if their metabolism is to be studied. In every case in which this has been done it has been found that the organisms grown *in vivo* behave differently metabolically than the organisms grown *in vitro*. The anthrax bacillus, the tubercle bacillus, or any other organism behaves differently metabolically when grown *in vivo*.

RODWELL: I was most interested in the paper by Tourtelotte and Jacobs. The correspondence in carbohydrate metabolism between their fermentative strains and the *mycoides* strains does indeed seem to be rather close. I, too, have estimated the products of glucose degradation during growth of *mycoides* with similar results. When grown in the partly defined medium I described elsewhere in this monograph, the products have varied with the growth conditions. I used the same three growth conditions described in my paper. Under the less strongly aerobic conditions, a large amount of lactate was formed, small amounts of pyruvate, and very small amounts of acetate whereas, in the strongly aerobic conditions, pyruvate accumulated as the major product. When grown in another medium, known in the laboratory as BVF-OS, a complex medium in which the organism grows very well and which is used as the standard medium for growth of pleuropneumonia vaccine, lactate was the major product under the less strongly aerobic conditions. Under aerobic growth conditions, both the glucose and lactate—small amounts of lactate are present in that medium—went to acetate. No pyruvate accumulated at all.

I am not quite sure how to interpret this. There might be a lesion in the pyruvic oxidase system in cells grown in the partly defined medium, or it might be reflection of some cell damage. Lactic dehydrogenase is one of the most active enzymes present in these cells. It may be that quite a large proportion of these cells is nonviable and that the pyruvate accumulation is produced by nongrowing, nondividing cells.

Neimark mentioned in his paper that we used cells suspended in water for our studies. When my wife and I started this work in about 1950 we came directly to it from work on metabolism of ordinary bacteria, so we began with the same methods. We soon found that, if we washed the cells, respiratory activity fell very rapidly but, in any case, the endogenous respiration was very low. Therefore, we simply used the sedimented paste suspended in water. I have since found that, if the cells are washed in buffered sucrose, about 0.4 molar, respiratory activity is maintained very much better. If manometric work is done in buffered sucrose, a $Q_{CO_2}(N)$ of about 1200 to 1500 is obtained on most substrates instead of our previous 300 or 400.

NEIMARK: I have some incomplete data on the heterofermentative strains under anaerobic conditions; these strains do shift toward much larger amounts of lactate. I, too, have tried suspending the organisms in 0.3 molar sucrose and obtained slightly better uptakes. One would suspect that the cells are lysing. I believe very strongly that the study of these organisms offers an extremely fertile field for understanding host-parasite relationships, especially as regards persistence in infections.

QUESTION: Neimark gave a comparison between the results he has found and the results that might be expected on bacteria. Would Lynn compare the character of the metabolic pattern that he discovered in PPLO with those known from bacteria?

R. J. LYNN (*University of Pittsburgh, Pittsburgh, Pa.*): I have not made these comparisons but, from what I have read on the subject, it would seem that the oxidative metabolism is somewhat comparable. This requires more study.

SMITH: We have reported that diphtheroids and the Campo strain of PPLO seem to be metabolically similar. In other words, the corynebacteria and many human strains of PPLO are similar with respect to oxidative metabolism.

COMMENT: In consequence of hearing that so much progress has been made on defining the metabolic requirements of this group of organisms, I strongly support Fabricant's statement that much work remains to be done in determining the nutritional requirements of various types of PPLO. When I was working extensively on the avian PPLO, including the pathogenic strains or the coccobacilliform bodies of Nelson, as far back as 1952, the problem was to differentiate the pathogenic coccobacilliform bodies and a vast number of nonpathogenic PPLO found in chickens with or without CRD. The only clue to a differentiation was that when employing a standard medium for PPLO, that is, Edward's medium, for culturing exudates from different kinds of respiratory diseases, a vast number of nonpathogenic PPLO could be isolated, but a vast number of the coccobacilliform bodies, which could be seen in smears of the exudate, failed to grow. It is sufficient just to emphasize how important the growth requirements are in leading to some very important findings. I am sure that, if this problem is investigated more critically, we shall be in a much better position to discuss pathogenic and nonpathogenic PPLO.

SMITH: I think we all agree that we cannot grow all the PPLO that we assume exist.

NEIMARK: The tissue culture work with *Brucella* by Pickett and Holland is somewhat similar. While Holland was working with these, he noted that he could put rough *Brucella* into tissue cultures and obtain smooth ones. Under certain sets of conditions, after putting in the rough *Brucella*, he was unable to obtain any growth, even by daily subculture for six days. I suggested that he try a rich medium, such as a serum containing osmotically supplemented medium: for example, PPLO broth or agar. Under these conditions Holland obtained smooth *Brucella* in three days. I think this illustrates the familiar condition that what can be cultivated depends upon the particular medium employed. Every time the nutritional level is raised, the next higher level of organisms can be cultured. There is reason to suspect that we are missing a large number of organisms in nature also.

Part V. Serology and Chemotherapy

PATHOLOGICAL, SEROLOGIC, AND CULTURAL CHARACTERISTICS OF TEN AVIAN STRAINS OF PLEUROPNEUMONIALIKE ORGANISMS*

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The condition now known as chronic respiratory disease (CRD) of chickens was first described by Delaplane and Stuart in 1943.[3] The agent isolated by Delaplane was egg-propagated.[4] Previously, Nelson[9] had reported on a coccibacilliform body as the cause of coryza of slow onset, which could be propagated in fertile eggs and tissue culture.

Markham and Wong[8] in 1952 successfully cultured the etiological agent of turkey sinusitis and CRD of chickens in cell-free media. Grumbles *et al.*[5] utilized carbohydrate broth enriched with pleuropneumonialike organisms (PPLO) serum fraction (Difco) in studying cultural and biochemical characteristics.

Jacobs *et al.*[7] and White *et al.*[10] described the use of a hemagglutination inhibition test for the PPLO group of organisms. Some PPLO did not hemagglutinate, and good hemagglutinating antigens could not be made from others; therefore, hemagglutination cannot be utilized in a serologic study of all organisms. Since the description of an agglutination test by Adler[1] in 1954, considerable progress has been made in the serologic study of PPLO. It is now possible to prepare antigens from almost any given strain of organism and compare it with other PPLO strains. Adler *et al.*[2] have shown that strains of PPLO differ serologically and in pathogenicity.

Materials and Methods

Antigen preparation. Antigens were prepared from each of 10 strains of PPLO by the inoculation of either phenol red dextrose broth enriched with 1 per cent serum fraction or serum-enriched chicken infusion media.[6] Five milliliters of a 24-hour culture were inoculated into 400 ml. of one of the media. The medium used for a particular strain was the one that gave most rapid growth. After inoculation, the medium was incubated at 37.5° C. for 48 hours. The resulting culture was centrifuged at 10,000 rpm for 30 min., and the sediment resuspended in buffered saline *p*H 7.0 and adjusted to 2 times No. 10 McFarland nephelometer. Enough formalin was added to the resuspended material to make a final dilution of 0.5 per cent, which was then stored at 4° C. for future use.

Preparation of antisera. Rabbits that gave negative reactions with previously prepared antigens were given subcutaneous inoculations with a washed

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suspension of the organisms without added formalin. Injections were made every third day for a total of six injections. Eight days after the last injection the serologic titer of each rabbit was determined, and those with adequate titers were then bled out; the serum collected from this blood was stored at −15° C. for future use.

Turkeys from a known PPLO-free flock were inoculated with chicken embryo cultures of the various strains of PPLO. Each of 3 turkeys was injected intrasinusly and intratracheally with a 0.5-ml. inoculum of infected egg material. An additional 1 cc. of infected egg material was inoculated intramuscularly into each bird. These turkeys were killed after 4 weeks' postinoculation observation, and blood was collected from each. The serum was extracted and frozen until used. Preinoculation and terminal titers of these sera were determined with each of the antigens prepared.

Chickens from a known PPLO-free flock were inoculated in a similar manner to prepare positive sera.

Pathological characteristics. As each strain of PPLO was isolated, its pathogenicity was determined by the pathological changes produced following intrasinus inoculation of susceptible turkeys.

Cultural characteristics. The cultural characteristics of each strain were determined by inoculation of serum fraction–enriched phenol red carbohydrates (dextrose, lactose, maltose, sucrose, and mannitol). The ability of the strains to ferment these carbohydrates and the average number of days necessary for fermentation to occur, as well as their rate of growth in Hofstad medium,[1] were recorded.

Viability of each strain of PPLO was maintained by culture at monthly ntervals in 6-day-old embryos.

Hemagglutination. The ability of each organism to agglutinate chicken erythrocytes was determined by using the antigen before the addition of formain. To 0.1 ml. of antigen in a 13- × 100-mm. tube, 0.5 ml. of 0.25 per cent chicken erythrocytes was added. The mixture was placed in a 37° C. water bath for 30 min.; it was then incubated in a refrigerator for 60 min. and the results recorded.

Results

TABLE 1 gives the cultural characteristics of the 10 strains of PPLO studied. Although there were some major differences between pathogenic and nonpathogenic cultures, there was no consistent, single, differentiating characeristic. Slow growth in carbohydrate media with a longer period of time equired for the fermentation of sucrose were the cultural characteristics that gave the best indication of a potential pathogenic strain. Apparently, the pathogenic strains grew more rapidly in Hofstad media than did the nonpathogenic strains.

The reactions on the plate test of the various antigens and the antisera made in rabbits, turkeys, and chickens are shown in TABLE 2. For the most part, the reactions of the antisera of the various species were very similar, xcept that the titers of the nonpathogenic strains in chickens were much lower han those of the turkey and the rabbit. The rabbit sera did give a slight reaction undiluted, which might indicate some crossover between pathogenic and nonpathogenic strains; however, the 1:4 dilution was negative.

In most cases the sera resulting from inoculation of the pathogenic strains reacted at a dilution of 1:32 or more, whereas the nonpathogenic strains reacted at a dilution 1:8 or less. The pathogenic strains in turkeys and chickens elicited a positive plate test to commercial antigen by the tenth day post-inoculation.

TABLE 1

CHARACTERISTICS OF PLEUROPNEUMONIALIKE ORGANISMS ISOLATED FROM CHICKENS AND TURKEYS

Strain	Source	Pathogenic	Hemagglutination ability	Carbohydrate fermentation*					Growth in Hofstad medium	Lethal effect in chicken embryo†
				D	M	L	S	Mt.		
T1	Turkey	Yes	+	+ (3)	+ (3)	—	+ (12)	—	Good	(13) Scattered mortality occurring throughout incubation period; usually a mortality of 40 to 60%
C1	Chicken	Yes	+	+ (4)	+ (5)	—	+ (4)	—	Good	(14) Same as T1, except that it occasionally kills 100% of embryos
C2	Chicken	Yes	+	+ (3)	+ (3)	—	+ (7)	—	Good	(14) Same as C1
C6	Chicken	Yes	+	+ (9)	+ (9)	—	+ (9 to 17)	—	Very good	(10) Same as C1
C5	Chicken	No	+	+ (1)	+ (1)	—	+ (1)	—	Very poor	(11) Only an occasional individual embryo killed
C8	Chicken	No	+	+ (2)	+ (2)	—	+ (2)	—	Poor	(11) No mortality
C7	Chicken	No	+	+ (1)	+ (1)	—	+ (1)	—	Poor	(11) Only an occasional individual embryo killed
C3	Chicken	No	+	+ (1)	+ (1)	—	+ (1)	—	Very poor	(11) Only an occasional individual embryo killed
388	Turkey	No	+	+ (6)	+ (4)	—	+ (7)	—	Poor	(11) Kills 100% of embryos within 48 hours; usually within 24 hours
388a	Turkey	No	+	?					Poor	(12) Scattered embryo mortality occurring throughout incubation period; usually kills 20 to 40% of embryos

* The letters D, M, L, S, and Mt. represent, respectively, dextrose, maltose, lactose, sucrose, and mannitol. The number in parentheses is the usual time in days for fermentation of each carbohydrate.

† Number in parentheses is the number of passages made in chicken embryos.

There were at least four serologic types or strains of PPLO among the isolates studied on the basis of the serum plate test. One strain of *Escherichia coli* gave a reaction with two of the nonpathogenic strains of PPLO. Two other strains of *E. coli* were checked, but they did not cross-react with any strain of PPLO. These results indicate that most, if not all, of the pathogenic strains of PPLO have common antigenic factors and can be detected with the available commercial antigens.

The gross pathological lesions that resulted from inoculation of the various agents are shown in TABLE 3. In every case the agent used to inoculate the birds had been through at least 12 egg passages and, in some cases, several artificial media cultural passages. One pathogenic strain (C1) produced only slight lesions after several passages, while another strain (T1), after 28 passages n culture and 14 passages in eggs, was still capable of producing characteristic esions. Strain C8 was highly pathogenic, especially in its affinity for the air sac and trachea. This strain produced severe respiratory symptoms, and 1 bird had pneumonia.

TABLE 2

SEROLOGIC RELATIONSHIP OF STRAINS OF PLEUROPNEUMONIALIKE ORGANISMS

Antigen	Antisera*											
	T1	C1	C2	C6	283–8	495–8	C5	C7	C8	388	388a	*E. coli* No. 1†
Pathogens												
T1	+	+	+	+	—	—	0	0	0	?^{1}‡	0	0
C1§	+	+	?	+	—	—	—	0	0	0	0	—
C2	+	+	+	+	—	—	0	0	?^{1}‡	0	0	0
C6	+	+	+	+	—	—	0	0	0	0	0	0
Commercial	+	+	+	+	+	+	0	0	0	0	0	0
Nonpathogens												
C3	0	—	—	—	—	—	0	—	+	—	—	0
C5	0	—	—	—	—	—	+	—	0	—	—	0
C7	0	0	0	0	0	—	0	+	+	0	0	0
C8	?^{1}‡	0	0	0	0	—	0	+	+	0	0	+
388	Not successful in attempts to prepare a suitable antigen											
388a	0	0	0	0	—	—	—	0	0	+	+	—
E. coli No. 1	0	0	0	0	0	0	0	+	+	0	0	+

* Strains checked, but found to be not related are indicated by 0; those not determined are shown by —.

† Rabbit sera only.

‡ ?^{1}, Reacted partially with serum undiluted only (rabbit only).

§ C1 antigen was not a good one; this may explain the variable reactions.

Discussion

There are no measurable cultural characteristics that will definitely separate pathogenic from nonpathogenic strains. Hofstad medium seems to give an ndication of pathogenicity by rapidity of growth, but this is difficult to evaluate and may not be true for all strains.

The separation of pathogenic strains from the several antigenic types of nonpathogens appears to be accomplished best by serologic techniques. The pathogenic strains seem to possess a common antigenic factor not shared by the nonpathogens.

By the use of PPLO-free turkeys for isolation procedures, the agglutination test may facilitate the diagnosis of a pathogenic strain. In all cases the birds used in this study became serologically positive before any signs appeared.

TABLE 3
GROSS LESIONS IN TURKEYS INOCULATED WITH THE VARIOUS STRAINS OF PPLO

Inoculum*	Bird No.	Exudate, sinus	Exudate, air sac	Exudate, trachea	Lung	Recovery of agent
Strain T1, C3, C11, T1, C28, E14, turkeys	1	3+	2+	–	Congested	+
	2	4+	4+–	–	–	
	3	1+	–	–	Edematous	
Strain C6, chickens, turkeys, E12	1	1+	4+	1+	–	+
	2	3+	4+	4+	–	
	3	1+	4+	4+	Pneumonia	
Strain C2, chickens, turkeys, C6, E16	1	1+	1+	–	–	+
	2	–	2+	1+	–	
	3	3+	2+	–	–	
Strain C1, chickens, C13, E16	1	–	1+	–	–	+ Air sac only
	2	–	1+	–	–	
	3	–	2+	–	–	
Strain C7 isolated from chickens, C13, E13	All birds	–	–	–	–	–
Strain 388A isolated from turkeys, E14	All birds	–	–	–	–	–
Strain C8 isolated from turkeys, C12, E12	1	–	–	–	–	–
	2	–	1+	–	–	–
	3	–	2+	–	–	–
Strain 388 isolated from turkeys, E13	All birds	–	–	–	–	–

* E, egg; C, culture; T, turkey.

Summary

The cultural, pathological, and serologic relationships of 10 strains of PPLO isolated from chickens and turkeys are reported. Differentiation of pathogenic and nonpathogenic strains may be accomplished by serologic procedures.

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USE OF THE HEMAGGLUTINATION-INHIBITION TEST IN THE CONTROL OF CHRONIC RESPIRATORY DISEASE OF CHICKENS

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This paper reviews the findings of my colleagues and myself on the use of the hemagglutination-inhibition (HI) test for detecting birds infected with chronic respiratory disease (CRD). This review does not take into consideration any of the saprophytic forms of the avian pleuropneumonialike organisms (PPLO) that may be found in the bird. We are concerned only with the particular organism that is invariably associated with CRD and is the underlying cause of the disease complex. The clinical disease in the bird does not occur without this organism but, on the other hand, it usually occurs with some other infective agent (a virus) that acts in an inciting role. Despite this, we have found that, if this organism is removed from the disease complex (whatever it may be), CRD does not occur. This of course leads one to the inescapable conclusion that the organism that causes hemagglutination is the real cause of CRD in chickens.

As a preliminary step in the development of the HI test, 30 strains of PPLO isolated from clinical cases of CRD in chickens and turkeys were tested for their growth and hemagglutinating properties. All of these strains produced hemagglutination, provided sufficient growth occurred. They were cross-tested against chicken sera from every province in Canada and from many areas in the United States, as well as against a few samples from the Caribbean area, England, France, Holland, Australia, and Israel. No essential differences were found, and one strain, designated D, which was isolated from a turkey and which produced the highest hemagglutinating titer, was selected for further work.

The earliest attempts at use of this strain in the HI test employed freshly grown broth cultures, and standardization was attempted through the use of 2 to 4 hemagglutination-antigen (HA) units, the HA unit being the least amount of antigen that would cause hemagglutination of a 1 per cent suspension of chicken red blood cells. It was soon found that this method was not sufficiently accurate for assay purposes; this was reflected in our inability to use such antigen for the critical detection of low levels of antibody. By referring to earlier work on the standardization of some of the toxoids, such as diphtheria and tetanus, a satisfactory method was developed. This method may be called a combining-power test, and it measures the potency of the antigen in terms of its combining power and that of a known standard: in this case, a dried reference serum prepared from the sera collected from infected broilers at the time of slaughter. The reference serum was Seitz-filtered, lyophilized in 2-ml. amounts, and sealed in the presence of nitrogen. Experience has shown that, when the titer of this reference serum is 1/40 (that is, 40 combining-power units), the antigen is of the desired concentration. This level is critical because antigen that is either too strong or too weak will give misleading results, particularly when one is dealing with low titers and wishes a test free of nonspecific reactions.

FIGURE 1 shows the results of a combining-power test. The antigen used is a stabilized and lyophilized preparation of PPLO antigen. It has been previously standardized to contain 40 C.P.U. We used a 3-dose assay spread 20 per cent apart, which seems suitable when twofold dilutions of serum are used. The test end points in the combining-power test are read at ++, that is, the irst tube to show any lessening in the agglutination caused by the antigen. Where we observe a drop from +++ to +, as in the 1/126 dilution, the end oint is read as occurring halfway between the 2 dilutions (that is, at 1/60 in his case).

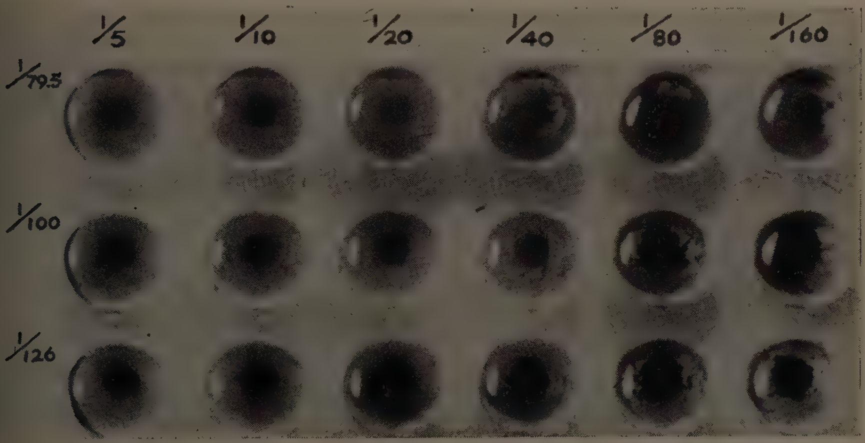

FIGURE 1.

TABLE 1
RESULTS OF A COMBINING-POWER TEST FOR PPLO

Antigen dilutions	Standard serum dilutions				
	1/5	1/10	1/20	1/40	1/80
1/79.5	–	–	++	+++	+++
1/100	–	–	–	++	+++
1/126	–	–	–	+	+++

In TABLE 1, the results shown in FIGURE 1 have been converted to symbols, nd it remains only to convert these symbols to figures. This is done most imply by arranging the titers of the 3 doses to the 1/100 dilution. Thus it is ossible to compare the combining power of the standard reference serum with he combining power of an unknown. The relative potency or the actual umber of test doses per unit of weight can be calculated directly. The accuracy of the method is approximately ±10 per cent.

In FIGURE 2 the results of testing 5 field sera at various dilutions are shown. For purely diagnostic purposes only 1 dilution need be used, namely the 1:5, and any serum causing complete inhibition at this dilution is considered to be positive.

In TABLE 2 we have the relationship between the time of infection and the development of HI antibodies. It may be seen that the first positives were found 9 days after infection, but that 29 days elapsed before all samples became positive; this was 3 weeks after clinical symptoms became general.

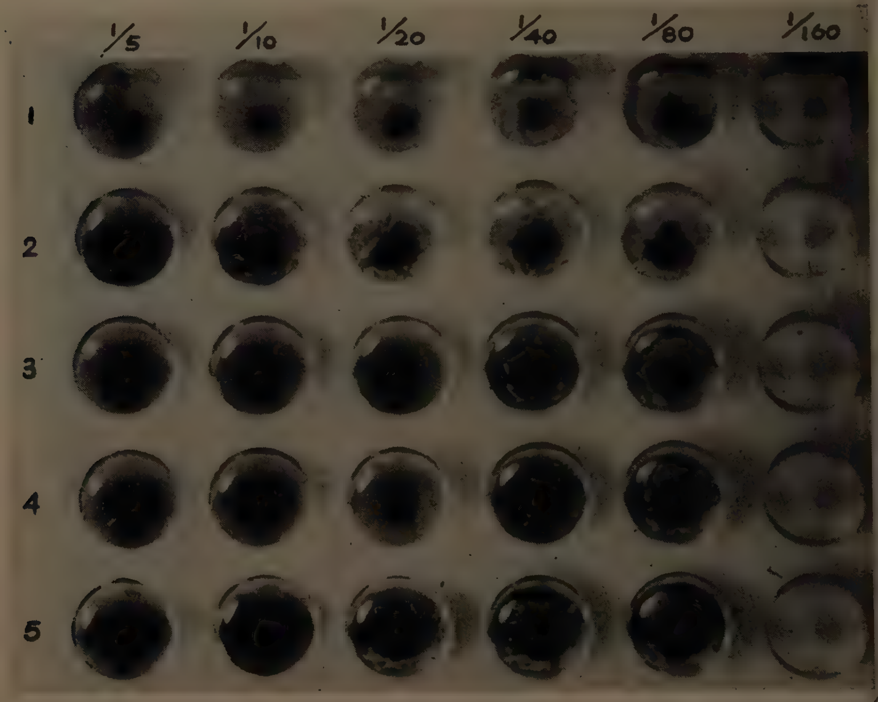

FIGURE 2.

In FIGURE 3 a graph is presented that shows the average HI antibody response after infection and the duration of these antibodies. It can be seen that detectable levels remain for at least 1 year and that they are in the range of 5 to 10.

In TABLE 3 similar results obtained from the artificial injection of turkeys are presented. In general the response is the same, but the HI titers tend to be higher. It is of interest to note that many birds have positive titers to this test, but have not had sinusitis. This apparent discrepancy is easily resolved if one accepts the idea that sinusitis is only one of the symptoms of CRD in turkeys.

In TABLE 4 are shown the results of testing 3200 flocks of chickens from various areas in Canada. These sera were collected mainly at blood-testing time and each serum sample was the result of taking several samples from the same

TABLE 2

DEVELOPMENT OF PPLO-HI ANTIBODIES IN BIRDS EXPERIMENTALLY INFECTED WITH CRD[*]

Clinical notes	Age (days)	Controls[†] (No. of birds)	HI titers[‡]									Titer of aliquot pool
			<5	5	10	20	40	80	160	320	640	
			(No. of birds in infected group)									
150 White Rock chicks plus 200 controls started	1	10	10									<5
Chicks inoculated intranasally	11	10	10									<5
Respiratory symptoms, all inoculated birds	20	10	8	2								<5
Respiratory symptoms present	26	10	6	1	3							5
Respiratory symptoms present	34	10	6	2	2							5
Respiratory symptoms present	40	10		1	1	2	4	2				40
Respiratory symptoms present	47	10				2	5	3				80
Respiratory symptoms present	54	10				1	5	4				80
Respiratory symptoms present	61	10				1	3	3	2	1		80
Lessening of respiratory symptoms	68	10				1	1	3	4	1		80
Symptom-free birds	75	10					1	5	2	1	1	160
Symptom-free birds	82	10					2	4	3	1		160
Symptom-free birds	89	10					3	3	3	1		160

[*] Inoculum consisted of bird-passage PPLO and CRD virus.

[†] Titers of less than 5.

[‡] Expressed as the reciprocals of the highest serum dilution causing complete inhibition of agglutination. Titers of less than 5 are considered to be negative.

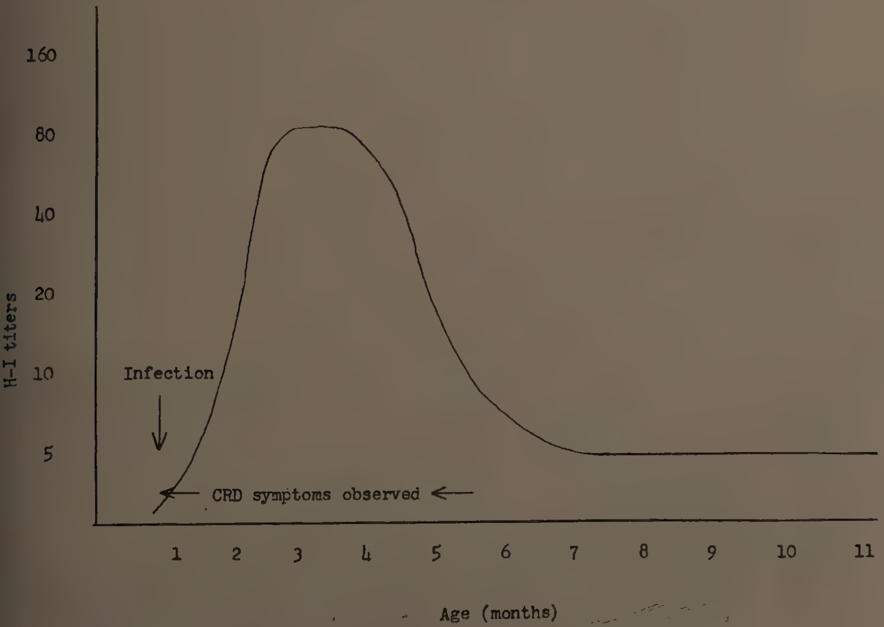

FIGURE 3. PPLO-HI antibody response in birds artificially infected with CRD.

flock. Admittedly, this is not the best sampling procedure, but at least it gives some idea of the prevalence of the disease in farm fields. It should be noted that almost all large commercial flocks gave positive responses.

The nature of the HI response in infected flocks is such that we feel that a sampling procedure will determine the CRD status of a flock. Experience has shown that the antibodies are so widespread in an infected flock that a testing procedure based on a maximum of 10 per cent will indicate whether infection is present. It is usual for 50 to 90 per cent of all birds in a flock to reach this level if infection is present. If infection is not present, there will be no reactions even at a 1:5 dilution.

TABLE 3
DEVELOPMENT OF PPLO-HI ANTIBODIES IN TURKEYS EXPERIMENTALLY INFECTED WITH PPLO*

Interval (days)	No. bled	HI titers								Titer of aliquot pool	No. of birds with sinusitis
		<5	5	10	20	40	80	160	320		
		(No. of birds)									
0	10	10								<5	0
8	10	3	4	3						5	6/19
15	19		2	5	8	1		2	1	40	16/19
22	10				3	2	2	2	1	80	17/19
Controls											
0	10	10								5	0
22	10	10								<5	0

* Inoculum consisted of sinus exudate obtained from turkeys with infectious sinusitis (PPLO only).

TABLE 4
INCIDENCE OF PPLO-HI ANTIBODIES IN CANADIAN POULTRY FLOCKS
(November 1953 to January 1957)

Province	Total of flocks	Serologic status		
		Negative	Positive	Percentages positive
Maritimes	173	68	105	60.7
Quebec	14	9	5	35.7
Ontario	1796	958	838	46.7
Manitoba	9	2	7	77.8
Saskatchewan	9	5	4	44.5
Alberta	931	519	412	44.2
British Columbia	292	127	165	56.5
Totals	3224	1688	1536	47.6

We think the HI test is a reliable and useful tool in establishing the CRD status of breeding flocks. However, it must be borne in mind that the antigen must be stabilized carefully if misleading and erroneous results are to be avoided.

THE THERMOSTABILITY OF CHICKEN SERUM TO BE USED IN THE PPLO AGGLUTINATION TEST*

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In the early research work on chronic respiratory disease (CRD) the task of producing an antigen from the pleuropneumonialike organisms (PPLO) was very difficult. However, many of the problems of growing these organisms on artificial media have been solved, thus facilitating the preparation of suitable antigens for serologic tests.

The need for a serologic tool in the diagnosis of CRD has long been established. In 1953 Jungherr[1] reported the results of experiments with a direct agglutination, hemagglutination, and a hemagglutination-inhibition (HI) test. The hemagglutination test that he used was patterned after that of Van Herick and Eaton.[2] Jungherr considered the HI test very useful. In this test the sera were inactivated at 56° C. for 30 min. Adler,[3] also in 1953, reported on the rapid-slide agglutination test and stated that this test appeared specifically to detect the presence of antibodies against the PPLO. The treatment of the sera in Adler's work was not indicated definitely in his report.

In the work of Jacobs *et al.*[4] with the HI test for PPLO antibody, the sera were inactivated at 56° C. for 30 min. and then stored frozen at $-20°$ C. The agglutination test, which was also included in these experiments, was reported as difficult to read; moreover, the results were inconclusive. Jungherr *et al.*[5] noted that inactivation at 56° C. for 30 min. nearly destroyed the antibody when the serum was used in the agglutination test, but slightly enhanced the readability of the HI test, suggesting that there is a difference in the two antibodies. Jungherr also suggested that serum could be stored satisfactorily without a preservative for 1 month at 4° C. and at $-20°$ C. for a 1-year period. The methods of handling the serum before use in the various serologic tests as reported by Dunlop,[6] Hofstad,[7] Crawley[8] and others were not indicated. In several experimental trials with the test at this laboratory (University of Delaware) some variations in the results were noted that were dependent upon the manner in which the serum was handled. The purpose of this paper is to report the results of several experiments designed to investigate the effect of refrigeration, freezing, and heating the serum to be used in the serum-plate agglutination and serum-tube agglutination test.

Experimental Procedures

The sera used in this experiment were obtained by venous or cardiac puncture of birds from three sources. Most sera were obtained from New Hampshire stock raised on the University of Delaware poultry farm. These birds had not shown any acute clinical signs of the disease, but when dead birds were examined they occasionally showed definite lesions of CRD. Other sera were

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* The work reported in this paper was conducted in cooperation with and supported in part by funds made available by the Animal Disease and Parasite Research Division, Agricultural Research Service, Department of Agriculture, Washington, D.C.

obtained from birds in the field that were showing definite clinical signs and lesions of CRD.

The antigens were obtained from the University of Connecticut, Storrs, Conn., and the American Scientific Laboratories (ASL), Madison, Wis. The Connecticut antigen was used in both the plate and tube tests, while the ASL antigen was used only in the plate test. In performing the tests, these antigens were used according to the directions of the manufacturer. The plate test was read at 2 min.

TABLE 1

RESULTS OF PPLO TUBE AGGLUTINATION TEST* AND NEWCASTLE HEMAGGLUTINATION TEST ON CHICKEN SERUM STORED AT 4° C. AND −15° C.

Sample number and time of storage (days)		Treatment of sample and results of tests			
		4° C. PPLO test	−15° C. Thawed once PPLO test	−15° C. Thawed each PPLO test	−15° C. Thawed each test HI titer
Serum I	1	1/50	1/200	1/100	1/5120
	2	1/50	1/100	1/200	
	4	1/100	1/200	1/200	
	8	1/200	1/200	1/200	
	32	1/200	1/200	1/200	1/640
Serum II	1	1/12.5	1/25	1/50	1/5120
	2	1/12.5	1/50	1/200	
	4	1/12.5	1/50	1/25	
	8	1/12.5	1/25	1/50	
	32	1/25	1/200	1/100	1/5120
Serum III	1	Neg.	Neg.	Neg.	1/640
	2	Neg.	Neg.	Neg.	
	4	Neg.	Neg.	Neg.	
	8	Neg.	Neg.	Neg.	
	32	Neg.	Neg.	Neg.	1/1280
Serum IV	1	1/50	1/50	1/25	
	2	1/25	1/25	1/12.5	
	4	1/50	1/50	1/200	
	8	1/50	1/50	1/100	
	32	1/50	1/50	1/200	

Key: Neg., negative.

* Highest dilution at which the serum produced 3+ or 4+ agglutination.

Dilutions of 1:12.5, 1:25, 1:50, 1:100, and 1:200 were used in the tube test, and the results were read after 20 to 26 hours' incubation at 37° C. The intensity of the reactions was recorded as negative, 1+, 2+, 3+, and 4+, the 4+ being complete agglutination. In the charts of this report only 3+ and 4+ reactions were considered as positive. In certain experiments the hemagglutination inhibition test for Newcastle disease, run according to the procedures of Cunningham,[9] was also included.

Experiment I. This experiment was designed to test the thermostability of sera after storage at 4° C. and −15° C. The frozen samples were divided into two aliquots, one of which was refrozen after each test, while the other was discarded. The sera were tested at intervals of 1, 2, 4, 8, and 32 days of storage. In this work a total of 15 sera was tested; each was obtained from birds grown on the University of Delaware poultry farm. The test results of a few typical reactions are shown in TABLE 1. The results indicated that the freezing of a positive serum increased its ability to react with the antigen. Negative sera, however, were not affected by the freezing and remained negative. The HI test for Newcastle disease antibody was not appreciably affected by storage.

Experiment II. The effect of heating the serum before it is used in the PPLO agglutination test was determined in this experiment. The sera were held in the refrigerator for approximately 2 days before being tested. Serum samples were exposed simultaneously to temperatures of 37° C. and 56° C. for intervals of 0, 10, 20, 30, 60, and 120 min. Typical results are shown in TABLE 2. Sera that showed some agglutinating power were not influenced by heating for as long as 120 min. at 37° C., but the activity of the serum was destroyed completely by heating at 56° C. for 10 min.

TABLE 2
RESULTS OF PPLO TUBE AGGLUTINATION TEST* ON CHICKEN SERUM INCUBATED AT 37° C. AND 56° C.

Sample number and duration of heat treatment (min.)		Temperature of incubation	
		37° C.	56° C.
Serum I	None	1/25	1/25
	10	1/25	Neg.
	20	1/25	Neg.
	30	1/25	Neg.
	60	1/25	Neg.
	120	1/25	Neg.
Serum II	None	Neg.	Neg.
	10	Neg.	Neg.
	20	Neg.	Neg.
	30	Neg.	Neg.
	60	Neg.	Neg.
	120	Neg.	Neg.

Key: Neg., negative.
* Highest dilution at which the serum produced 3+ or 4+ agglutination.

Experiment III. The effect of storing the serum at 4° C. or −15° C. followed by heating also was determined. A total of 13 samples of serum was tested. In this experiment the samples were heated for 20 or 80 min. at temperatures of 37 or 56° C. The typical results are shown in TABLE 3. Similar results were obtained in this experiment as in the previous two. Freezing the serum increased its ability to produce agglutination. The effect of heat on the sample was the same as indicated in Experiment II. Previous treatment of the serum, freezing, or refrigeration had no influence on the effect of heating the sample.

Since the agglutinating power of the sera, except for one sample, was completely lost by heating for 10 min. at 56° C., 15 additional sera were subjected to this temperature at time intervals of 0, 10, 20, and 40 min. Each serum

TABLE 3

RESULTS OF PPLO TUBE AGGLUTINATION TEST* ON CHICKEN SERUM INCUBATED AT 37° C. AND 56° C. AND THE HEMAGGLUTINATION-INHIBITION TEST ON SERUM INCUBATED AT 56° C.

Sample number and test applied	Treatment of aliquot samples									
	Stored at 4° C.					Stored at −15° C.				
	No heat	37° C.		56° C.		No heat	37° C.		56° C.	
		20 min.	80 min.	20 min.	80 min.		20 min.	80 min.	20 min.	80 min.
Sample I										
PPLO test	1/50	1/100	1/50	1/12.5	1/25†	1/200	1/100	1/100	Neg.	Neg.†
HI test	1/5120	—	—	—	1/20	—	—	—	—	1/20
Sample II										
PPLO test	Neg.	Neg.	Neg.	Neg.	Neg.†	Neg.	Neg.	Neg.	Neg.	Neg.†
HI test	1/5120	—	—	—	1/40	—	—	—	—	Neg.
Sample III										
PPLO test	1/12.5	1/12.5	1/12.5	Neg.	Neg.†	1/25	1/50	1/12.5	Neg.	Neg.†
HI test	1/640	—	—	—	1/5	—	—	—	—	1/5
Sample IV										
PPLO test	1/25	1/25	1/50	Neg.	Neg.†	1/50	1/50	1/50	Neg.	Neg.†
HI test	1/1280	—	—	—	Neg.	—	—	—	—	Neg.

Key: Neg., negative.

* Highest dilution at which the serum produced 3+ or 4+ agglutination.

† Heating serum at 56° for 80 min. produced cloudiness of the serum; therefore the results are questionable.

TABLE 4

EFFECT OF HEATING SERUM SAMPLES AT 37° C. OR 56° C. AFTER STORAGE AT 4° C. OR −15° C. BEFORE USE IN THE PPLO TUBE AGGLUTINATION TEST* AND IN THE HEMAGGLUTINATION-INHIBITION TEST FOR NEWCASTLE DISEASE

Sample number	Stored at 4° C.						
	No heat		Heated at 56° C. for				
			10 min.		20 min.		40 min.
	PPLO	HI	PPLO	HI	PPLO	HI	PPLO
B	Neg.	1/640	Neg.	1/640	Neg.	1/640	Neg.
C	1/25	1/640	Neg.	1/640	Neg.	1/640	Neg.
D	Neg.	1/640	Neg.	1/640	Neg.	1/640	Neg.
G	1/50	1/640	Neg.	1/640	Neg.	1/640	Neg.
K	1/25	1/320	Neg.	1/320	Neg.	1/320	Neg.

Key: Neg., negative.

* Highest dilution at which the serum produced 3+ or 4+ agglutination.

was previously divided into two samples, one stored at 4° C. and one at −15° C. The hemagglutination titers for Newcastle disease were also determined in parallel at the same temperatures for a comparison. The results shown in TABLE 4 indicated that incubation after 10 min. at 56° C. completely destroyed the ability of the serum to react, but this treatment did not influence the HI titer for Newcastle disease. The Newcastle antibody also was unaffected when the serum was incubated for 20 min. at 56° C.

Experiment IV. The effect of centrifugation of the serum following storage at 4° C. and −15° C. on the results of the PPLO agglutination test also was determined. Sera were divided into two aliquots, one of which was centrifuged at 2000 rpm for 10 min. After centrifugation the serum for the test was taken from the center of the sample, care being taken not to disturb the sediment or the upper layer. The results shown in TABLE 5 indicate that the centrifugation of samples has very little if any influence on the reaction in the tube agglutination test. A total of 31 serum samples was tested.

TABLE 5
RESULTS OF PPLO TUBE AGGLUTINATION TEST* ON CHICKEN SERUM SAMPLES STORED AT 4° C. AND −15° C.

Sample number and days of storage	Treatment of sample			
	4° C.		−15° C.	
	Centrifuged	Not centrifuged	Centrifuged	Not centrifuged
I—1	1/12.5	1/25	1/50	1/50
2	1/12.5	1/25	1/100	1/25
8	1/12.5	1/25	1/25	1/25
32	1/12.5	1/50	1/50	1/100
II—1	1/25	1/25	1/100	1/50
2	1/50	1/25	1/50	1/25
4	1/25	1/50	1/50	1/50
8	1/50	1/50	1/100	1/50
16	1/50	1/50	1/50	1/50
III—1	1/50	1/100	1/100	1/200
3	1/50	1/100	1/200	1/100
6	1/100	1/100	1/200	1/200
8	1/200	1/100	1/200	1/200
10	1/200	1/200	1/200	1/200

* Highest dilution at which the serum produced 3+ or 4+ agglutination.

Experiment V. The serum plate test was performed in parallel with each tube agglutination test to determine the uniformity of the reactions. The esults of the plate test on serum samples that were stored at −15° C. were imilar to those of the tube test with one notable exception. Thirteen of 27 amples that were negative prior to freezing became 3+ or 4+ reactions.

In the plate test the sera were more resistant to heating at 56° C. in that 10 of 27 samples still retained agglutinating activity after incubation for 80 min. at this temperature.

Experiment VI. In general the Connecticut and ASL antigens compared very favorably in respect to the results obtained with aliquots of the same serum. In some samples the Connecticut antigen produced stronger positive results than the ASL antigen. A few examples of the test results are shown in TABLE 6.

TABLE 6

RESULTS OF SERUM OR PLASMA PLATE AGGLUTINATION TESTS WITH THE CONNECTICUT AND ASL ANTIGENS

Sample number and type	Storage			
	4° C.		−15° C.	
	Reaction with antigen		Reaction with antigen	
	Conn.	ASL	Conn.	ASL
1. Plasma	4+	3+	4+	4+
1. Serum	4+	4+	4+	4+
2. Plasma	4+	3+	4+	4+
2. Serum	4+	4+	4+	4+
3. Serum	3+	1+	4+	2+
4. Serum	2+	1+	4+	2+
5. Serum	4+	2+	4+	2+
6. Serum	1+	Neg.	3+	2+
7. Serum	Neg.	Neg.	Neg.	Neg.

Key: Neg., negative.

Summary

These experiments indicated that:

Freezing the serum used in the PPLO tube or plate agglutination test increased the ability of a positive serum to agglutinate the antigen.

The duration of the storage in the frozen state had no effect on this reaction.

In the tube agglutination test, heating the serum at 37° C. for as long as 120 min. did not influence the agglutinating power of the serum, but heating it for 10 min. at 56° C. completely destroyed the activity of the sample except in one case.

Centrifugation of the serum did not alter the readability of the test under the conditions of this experiment.

Serum samples that did not show agglutinating properties by the tube test were not affected by the freezing or by the heating used in this experiment.

Serum samples stored at 4° C. for as long as 32 days did not lose their ability to produce agglutination of the antigen.

The results of the serum plate test indicate that there is a disagreement between this test and the tube test with regard to the effect of freezing and the effect of heating (56° C.).

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COMPARISON OF PPLO STRAINS FROM TISSUE CULTURE BY COMPLEMENT FIXATION*

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In this report we describe the results of culturing all of our cell lines and those from several other laboratories for the presence of PPLO, together with preliminary results of antigenic analysis of the strains by means of the complement fixation test.

As reviewed by Dienes,[1] Edward,[2] Klieneberger-Nobel,[3] and others, pleuropneumonialike organisms (PPLO) grow well in the presence of media rich in animal protein or serum, or in the presence of living cells. Penicillin[4,5] and other antibiotics[6] have been shown to favor the isolation of PPLO, in some cases to stimulate their growth and to cause or aid in the derivation of PPLO or L forms from bacteria. Tissue culture media provide all of these conditions favorable for the growth of PPLO. Furthermore, the presence of PPLO in tissue cultures does not produce turbidity of the medium or other readily apparent gross change. There are no reliable microscopic criteria for recognizing the presence of PPLO in tissue cultures, and they do not grow when sterility tests are made in the usual bacterial media. Several published reports[7-9] and others elsewhere in this monograph suggest that PPLO may indeed be a common unrecognized contaminant of continuous-culture cell lines.

Materials and Methods

All PPLO cultures were made by George Rothblat, Department of Medical Microbiology, University of Pennsylvania, by methods described elsewhere.[9] Cell cultures were removed from the glass container with 0.25 per cent Versene, centrifuged, and the pellet of cells frozen at −20° C. until they were inoculated into Bacto beef-heart infusion broth containing 1 per cent Bacto-PPLO serum fraction. At intervals the broth cultures were subcultured to plates of Bacto-PPLO agar and examined under the microscope for PPLO colonies. Cultures of cell lines received from other laboratories were made from the original tube to obviate the question that contamination might occur in our laboratory on passage.

PPLO antigens for complement fixation were grown in liquid PPLO medium by Paul Smith (University of Pennsylvania), collected in a Sharples centrifuge, washed 3 times with saline, suspended in saline, and a Klett-Summerson photoelectric colorimeter reading compared with a standard, previously determined by a micro-Kjeldahl to determine the mg. of N/ml. All antigens were standardized to contain 0.025 mg. N/ml. by diluting with Kolmer saline. White New Zealand rabbits, weighing from 4 to 6 pounds, were immunized with the standard suspension of live PPLO mixed with an equal amount of 4 per cent aluminum hydroxide gel adjuvant.[10] Each rabbit received an initial dose

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of 0.1 ml. subcutaneously, followed by gradually increasing doses every other day for 2 weeks. This was followed by a final injection of 1 ml. at 21 days. The rabbits were bled at 28 days and the serum stored in sterile vials without preservative. All sera were heat-inactivated at 60° C. for 30 min. just before use. A modified Kolmer technique[8] was used with overnight fixation and 2 exact units of complement titrated in the presence of antigen. End points were read at the highest dilutions showing a plus 3 or plus 4 fixation of complement. Controls consisted of PPLO medium with and without serum fraction, pooled normal rabbit serum, and prebleeding sera of all immunized rabbits, as well as serum controls and antigen controls.

TABLE 1

Lab. No.	Negative	Positive
Number of HeLa Cell Lines Cultured for PPLO		
1	2	
2	2	
3	1	
4	2	
5	1	1
6	1	2
8		1
10		1
11		1
Subtotal	9	6
Number of Other Cell Lines Cultured for PPLO*		
5	1	1
6	7	3
7		2
9		3
Subtotal	8	9
Total 11	17/32 = 53%	15/32 = 47%
7/11 Positive = 64%		

* Positive cell lines other than HeLa were: SJD, Chang's conjunctiva, human intestine, Chang's kidney, human lung, MCN, L cell (not from Wilton Earle's laboratory), MK, C_3, and HEp 2.

Results

Of 32 cell lines received from 11 laboratories, 15 were positive for PPLO, or 47 per cent of all cell lines examined, as shown in TABLE 1. One or more cell lines from 7 of the 11 laboratories were positive, or 64 per cent of the laboratories.

Rabbit antisera were prepared against a representative PPLO, T5, isolated from one of our HeLa cell lines and against a stock laboratory strain, 07, from human genital origin, Type 2, supplied by Morton.[11] The complement fixation titers of these antisera with 13 of the 15 PPLO strains from cell cultures are shown in TABLE 2. The cell culture PPLO strains reacted only in low titer with the O7 antiserum, the highest titer being thirty-twofold less than the serum titer with the homologous O7 antigen. This suggests that none of the cell culture PPLO strains belongs to human genital Type 2. All 13 strains did react in high titer with the T5 antiserum; 9 equal to, or 1 tube lower than, the homologous titer, and 4 within fourfold dilution of the homologous titer. This suggests that all PPLO strains isolated from cultures are closely related antigenically.

TABLE 2
COMPLEMENT FIXATION TITER OF ANTISERA

TC PPLO antigen	CF titer of antiserum versus	
	O7	T5
O7*	1024	16
HeLa (T5)	16	128
HeLa	16	64
Conjunctiva	16	32
Human intestine	16	64
Chang's kidney	32	128
Lung	16	32
Human bone marrow	16	64
L cell	8	32
Monkey kidney	32	32
Monkey kidney	16	64
HeLa	32	128
Human thyroid	32	128
HeLa	32	128

* This PPLO, O7, is a laboratory stock strain belonging to human genital Type 2.

TABLE 3
COMPLEMENT FIXATION WITH NONTISSUE CULTURE PPLO

PPLO	Source	Antisera versus	
		O7	T5
Campo	Human Type 2, genital	512	8
Cat	Cat	16	64
J	Chicken	32	8
Calf	Calf	128	8
V-73	Human genital	16	64
O7	Human Type 2, genital	1024	16
T5	TC	16	128
PG 25	Human Type 1, genital	32	64
PG 20	Human Type 4, oral	16	16

These two antisera also were tested with PPLO antigens from nontissue culture sources, and the results are shown in TABLE 3. It is apparent that the antiserum against Type 2, laboratory stock strain O7, reacts with both O7 and Campo, but only in low titer with a PPLO stock strain of human genital Type 1, a human oral Type 4, and with strains from a cat, a chicken, and a calf, and e tissue culture strain T5. The antiserum against strain T5 reacts strongly nly with antigen from human genital Type 1, with the strain isolated from a at, and with strain V-73 of human genital origin. This latter strain is pre- umably a human Type 1 strain. The evidence suggests that the tissue culture rains were derived from a human genital Type-1 source or sources.

In TABLE 4 the results of tests with antisera against stock strains of human ypes 1 and 4 indicate that none of the tissue culture strains is related to Type However, 6 of the 8 cell culture strains react in high titer with antisera to uman Type 1. One PPLO strain from an L-cell subline and one from a HeLa ibline reacted in lower titer with this serum. This is the only evidence in l our complement fixation studies indicating that some of the cell culture PLO strains may be different from the others.

TABLE 4

COMPLEMENT FIXATION WITH ANTISERA AGAINST HUMAN PPLO TYPES 1 AND 4

PPLO antigen	Antisera	
	Type 1 PG 25	Type 4 PG 20
PG 25(1)	64	8
PG 20(4)	8	64
07(2)	8	16
T5	64	8
Human thyroid	64	0
HeLa ST-C	64	0
HeLa C-2-7	8	8
Human intestine	32	8
L cell	16	0
Monkey kidney	32	8
Human lung	32	8

All of the above results indicate that the labeled stock strains were correctly signated, and that the complement fixation system used was sensitive and iable in detecting antigenic differences and similarities of these known strains. may be assumed that the same reliability applies to the unknown strains, and erefore that the T5 strain of PPLO is representative of the majority of PPLO ains isolated from 7 tissue culture laboratories. The relationship of the LO strains from cell culture to the other known strains that we have been e to study can, therefore, be represented by the reactions of T5 in cross- nplement fixation tests shown in TABLE 5.

Source of PPLO in Tissue Cultures

ecause PPLO are fairly widespread in nature, one may postulate many ential sources from which cell cultures may be contaminated. It has been nonstrated that all human types can grow in tissue cultures. Some of the ious potential sources for infection are as follows:

1) Saliva or droplets from the technicians handling cell cultures. This ms unlikely, since none of the recovered strains were related antigenically to human oral Type 4 strain.

(2) Fingers and fomites that might transmit human genital or oral strains. This also seems unlikely because most of the strains isolated were antigenically similar, and it would be unusual for the same strain to be picked up by chance in each of 7 widely separated laboratories in the United States and Canada. Furthermore, the tissue culture PPLO strains were not related to the genital Type 2 commonly found in this country, but to the genital Type 1 which has been most frequently isolated in England.

(3) Serum or other ingredients of the tissue culture media might introduce the organisms. Cultures of the following media for PPLO proved to be negative: 20 lots of human sera, 1 horse serum that had been used for months in our laboratory, 2 lots of calf serum and 2 lots of bovine serum, 6 rabbit sera, and 3 fresh human tumors. However, Christenson has isolated PPLO from cow serum and from horse serum collected at a slaughterhouse.[12]

(4) We have speculated whether PPLO might be related to malignancy or favor establishment of cell lines in serial passage. After extensive trials we have not been able to establish monkey kidney cells in serial passage by infection with T5 PPLO. Neither were monkey kidney cells transformed by PPLO so that they would produce tumors in cortisone-X-ray-treated rats.

TABLE 5

CROSS-COMPLEMENT FIXATION TESTS OF PPLO STRAINS

Antisera	Type	Antigen			
		Human Type 1 (PG 25)	Human Type 2 (O7)	Human Type 4 (PG 20)	Cell-culture type (T5)
PG 25	1	$\boxed{64}$	8	8	64
O7	2	32	$\boxed{1024}$	16	16
PG 20	4	8	16	$\boxed{64}$	8
T5	TC	64	16	16	$\boxed{128}$

(5) Bacteria falling into tissue cultures might be suppressed by the antibiotics, but give rise to PPLO or L forms. It does not seem probable that all PPLO strains so derived would belong to a single antigenic type unless all PPLO contain common antigens that arise from a common constituent of bacterial cells. By means of special tests a Gram-positive diphtheroid was found in one of our cell lines that also contained a tissue culture-type PPLO. At another time, a Gram-positive rod with a yellow pigment was found in the stock supply of calf serum and in 13 cell lines fed with this serum. Neither of these contained antigens that gave significant cross reactions in complement fixation tests with antisera for any of the PPLO strains available. On the other hand, bacterial forms were isolated from pure cultures of tissue culture PPLO by Rothblat on several occasions. Eighteen of these strains of bacteria derived from PPLO cultures were tested for complement fixation with T5 and O7 antisera. Eleven reacted in very low titer with T5 antisera, and 12 reacted with O7 antisera, 3 giving a titer of 1 to 32. None reacted with antisera for human PPLO Types 1 and 4. Rabbit antisera were made for 3 of the more active bacterial antigens

and were cross-reacted with all bacteria and the standard PPLO reagents. Results showed that (1) the majority of the bacterial strains shared common antigens, and (2) bacterial antisera did not fix complement with any PPLO antigens, but O7 and T5 PPLO antisera did bind complement in low dilution with some of the bacterial antigens. This needs further study. It suggests that the bacterial forms might have been derived from the tissue culture PPLO. If this is true, the PPLO may have been derived originally from bacterial contaminants.

(6) Another possible source of cell culture PPLO is through exchange of cell lines between tissue culture laboratories. The data presented above prove that this can and does occur. This would offer a simple explanation for the antigenic similarity of PPLO strains from many laboratories and for the fact that most strains belong to an antigenic type that is uncommon in the United States. It would not explain the source of the original contamination. However, since PPLO are normally found in the female genital tract,[11] cell lines derived therefrom may well have contained PPLO from the beginning or have been infected early by feeding with human placental serum.

Removal of PPLO from tissue culture cell lines has proved to be very difficult. We have tried terminal dilution and various antibiotics in maximal doses tolerated by the tissue culture cells. Terramycin is effective in suppressing the cell culture PPLO, but after several transfers in its absence the PPLO reappears. One HeLa strain was permanently freed of PPLO by transferring it each week for 6 weeks in the presence of 4 μg./ml. of Terramycin and feeding once between each transfer with 8 μg./ml. of Terramycin. We have not completed studies with hyperimmune sera, which might be useful for this purpose. Antiserum is quite effective in killing PPLO growing in artificial media.[13]

Discussion

Repeated cultures of a PPLO-infected tissue cell line are not always positive; we may therefore assume that the incidence of PPLO in tissue culture laboratories is greater than is indicated by the data presented here. The effects of PPLO on tissue culture cells have been explored only slightly. We have some evidence that their presence is not related to malignancy or to transformation or establishment of altered cell lines, or to susceptibility of poliovirus infection. These and the effects on metabolism, enzymes, nutritional requirements, and antigenic structure need further study because the presence of PPLO may invalidate otherwise careful studies. A rapid method for detection of infection and effective procedures for elimination or prevention of infection would be most helpful.

Finally, the widespread prevalence of PPLO and bacterial infection in tissue culture laboratories is somewhat analogous to the prevalence of antibiotic-resistant staphylococcus in hospitals. Both develop and persist as the result of excessive use of antibiotics, which permits adaptation and mutation of resistant organisms and encourages poor aseptic technique because breaks and errors are covered up or masked by the antibiotics. The remedies also may be similar in part, such as less widespread use of antibiotics and return to rigid aseptic techniques. It would seem wise for tissue culture laboratories to carry all stock cultures of cells without antibiotics. Antibiotics could be added for mass cultures and numerous other procedures that are impossible without antibiotics. It also would seem wise to incorporate a culture for the presence of PPLO as a routine sterility test for cell lines.

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THE EFFECTS OF ANTIBIOTICS ON THE INFECTIOUS SINUSITIS AGENT OF TURKEYS: THE *IN VIVO* DEVELOPMENT OF ANTIBIOTIC-RESISTANT STRAINS OF *MYCOPLASMA**

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Several authors have reported on the effectiveness of antibiotics against the pleuropneumonialike group of organisms (PPLO) as isolated from chickens and turkeys.[1,3-6,8,9] Most of these workers agree that streptomycin, dihydrostreptomycin, chlortetracycline, oxytetracycline, erythromycin, chloramphenicol, carbomycin, and viridogrisein are more effective against these isolates than are penicillin, neomycin, and bacitracin. Penicillin is used almost routinely in the isolation of *Mycoplasma* from birds, so that one may logically conclude that this antibiotic does not have a significant inhibitory capacity for these organisms. The antibiotics used in the experiment described below were selected from antibiotics that have been demonstrated by other workers to be effective against chicken and turkey isolates of PPLO. Fahey[2] has previously reported a field outbreak of infectious sinusitis due to PPLO resistant to streptomycin and partially resistant to oxytetracycline and chlortetracycline.

The purpose of this paper is to present quantitative data concerning the production of antibiotic-resistant strains of PPLO by oral and parenteral antibiotic therapy. These antibiotic-resistant strains persisted in the adult hens and were also egg-transmitted to their progeny. The effectiveness of antibiotic therapy in preventing egg transmission of the infectious sinusitis agent of turkeys has been reported by us.[7]

Materials and Methods

A natural outbreak of infectious sinusitis occurred in a flock of 116 Beltsville small white turkeys housed at the experiment station at the University of Minnesota. The disease was diagnosed by clinical and bacteriological examination of the flock. Almost all of the birds developed respiratory rales with dyspnea; in addition, 54 per cent developed enlarged infraoribtal sinuses. Four birds were sacrificed and their tissues collected for isolation of the agent. Tracheas, lungs, and air sacs were triturated and inoculated into 7-day-embryonated chicken eggs via the yolk sac route. Aspirated sinus exudate was also inoculated into embryonated eggs by the same route. Twelve isolations of PPLO were obtained. The isolates were cultured in semisynthetic media containing PPLO broth WO/CV (Difco 0554-01) with 1 per cent enzymatic yeast and 2 per cent PPLO bovine serum fraction (Difco 0441-63). Thallium acetate was added at a level of 0.5 gm./l. and penicillin G included at 500,000 I.U./l. The isolates, also cultured in phenol red carbohydrate media similarly enriched, grew quite well; they fermented dextrose and maltose within 72 hours, while sucrose was delayed. Lactose and mannitol were not fermented. The flock was divided into 9 groups, 8 of which were treated with antibiotics for 5 months, as indicated in TABLE 1.

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At the termination of the treatment, PPLO isolates from each treated group were tested for resistance and cross resistance to antibiotics and compared with the isolates of PPLO obtained from the flock prior to treatment. The same procedure was followed with the isolates obtained from the poults hatched from the hens receiving antibiotic therapy.

The sensitivity to antibiotics of the PPLO isolates was determined by a twofold serial dilution technique. The test medium was the same as that used in the original isolation, except that 1 per cent PPLO bovine serum fraction was used in lieu of a 2 per cent concentration. The antibiotics tested included dihydrostreptomycin sulfate, tetracycline hydrochloride, chlortetracycline hydrochloride, oxytetracycline hydrochloride, and erythromycin lactobionate. All solutions of antibiotics were made immediately prior to use to prevent any inactivation, and 0.5 ml. of the antibiotic solution of each concentration of antibiotic was added to 4.5 ml. of test medium to give a final concentration of from 800 to 0.39 μg./ml. of medium. For an inoculum, 0.1 ml. of a 72-hour culture of each respective isolate was used. A tube containing 5 ml. of media was also seeded to serve as a control. All tubes were then incubated at 37° C. for 6 days. At the end of incubation, or as soon as turbidity had occurred in the tubes, an aliquot from each tube was examined by phase microscopy. A tube of thioglycollate medium, and a tube of enriched phenol red maltose medium were inoculated with 0.5 ml. from each tube. Blood agar plates were streaked with a loop of material from each tube demonstrating turbidity. These procedures were carried out to exclude the possibility of bacterial contamination and to establish the presence of PPLO.

TABLE 1

TREATMENT OF BREEDING TURKEYS WITH VARIOUS ANTIBIOTICS*

Pen No.	Antibiotic†	
	In feed	Injected
I	Control	Control
II	TC 1000 gm./ton feed	DHS 50 mg./lb. I.M.
III	TC 1000 gm./ton feed	None
IV	None	CTC 5 mg./lb. I.V.
V	CTC 1000 gm./ton feed	CTC 5 mg./lb. I.V.
VI	None	OTC 5 mg./lb. I.V.
VII	OTC 1000 gm./ton feed	OTC 5 mg./lb. I.V.
VIII	None	DHS 50 mg./lb. I.V.
IX	EM 1000 gm./ton feed	EM 50 mg./lb. I.V.

* All intramuscular (I.M.) and intravenous (I.V.) injections were given every 48 hours (for the first 3) and once weekly thereafter.

† Key to abbreviations: TC, tetracycline (HCl); CTC, chlortetracycline (HCl); OTC, oxytetracycline (HCl); DHS, dihydrostreptomycin (sulfate); and EM, erythromycin (lactobionate).

The point of minimum inhibitory concentration (MIC) was defined as the least concentration of antibiotic that would prevent visible growth of the organism for six days. The degree of antibiotic resistance was obtained by dividing the MIC of the isolate following treatment of birds with the MIC before antibiotic treatment.

Several controlled field trials have been initiated in an effort to determine if antibiotic resistance can occur following short-term antibiotic therapy. First the agent was isolated, and then the flocks were mass-treated with a single antibiotic. After the antibiotic therapy the unrecovered birds were divided into groups and treated with several antibiotics in an effort to determine which would be most effective in these circumstances. The PPLO agent isolated after therapy was compared to that isolated before therapy in regard to antibiotic resistance. Several flocks also have been followed in the same manner except that no isolate could be made prior to the single antibiotic treatment as the owner had treated the flock before it was brought to our attention. Isolates of PPLO were then made before the birds were re-treated with the other antibiotics.

TABLE 2

THE *IN VITRO* ANTIBIOTIC SENSITIVITY OF STRAINS OF PPLO ISOLATED FROM THE ANTIBIOTIC-TREATED ADULT TURKEYS COMPARED TO THE ORIGINAL ISOLATE

Turkey group isolate	Antibiotic pretreatment*	Minimum inhibitory concentration (µg./ml.)					
		Streptomycin	Dihydrostreptomycin	Tetracycline	Chlortetracycline	Oxytetracycline	Erythromycin
I A	None (control)	3.13	6.25	1.56	1.56	0.78	100.00
II A	Dihydrostreptomycin Tetracycline	12.50	50.00	6.25	6.25	1.56	100.00
III A	Tetracycline	3.13	6.25	100.00	25.00	25.00	100.00
IV A	Chlortetracycline	3.13	6.25	50.00	200.00	50.00	100.00
V A	Chlortetracycline	3.13	6.25	50.00	200.00	50.00	100.00
VI A	Oxytetracycline	3.13	6.25	50.00	100.00	100.00	100.00
VII A	Oxytetracycline	3.13	6.25	50.00	100.00	100.00	100.00
VIII A	Dihydrostreptomycin	100.00	400.00	1.56	0.78	1.56	100.00
IX A	Erythromycin	3.13	6.25	1.56	1.56	0.78	100.00
Original isolate	(Pool of isolates)	3.13	6.25	1.56	1.56	0.78	100.00

* See TABLE 1 for antibiotic treatment protocol.

Results

Prolonged therapy experiment. Of the PPLO isolated from the adult hens and from poults hatched from these hens, only one isolate failed to develop antibiotic resistance. TABLE 2 lists the MIC of antibiotic found necessary to inhibit the strains isolated from the adult turkey hens following therapy. These isolates are compared with the MIC of the original isolate (pooled isolates) prior to antibiotic therapy. The data presented in TABLE 2 indicate that, although the original isolate was not particularly sensitive to erythromycin, no increased resistance to this drug was developed following therapy. It may be observed that antibiotic resistance following therapy was found in all other isolates derived from all adults but those in the control group (I A, TABLE 2).

TABLE 3 lists the MIC of antibiotics necessary to inhibit growth of the strains isolated from poults hatched from the treated hens. The data presented (TABLE 3) indicate that the antibiotic-resistant strains produced in the adult hens after prolonged antibiotic therapy were egg-transmitted to their progeny. These results also indicate that the dihydrostreptomycin-resistant strain was infective for other birds, as this strain infected the control group of poults (I P, TABLE 3), as well as two other groups. This observed cross infection occurred seemingly because of inadequate isolation of groups during brooding. No clinical signs were observed in the poults; however, on post-mortem examination the poults were found to have lesions within the air sacs. The antibiotic-resistant isolates obtained from the adult birds and poults when inoculated into experimental birds did not produce the typical clinical syndrome of infectious sinusitis in the inoculated birds. The isolates were of decreased virulence, as only a serologic response was obtained in the inoculated birds. The sera of the inoculated birds reacted with a plate antigen prepared from the original isolate and an antigen prepared from a strain of PPLO known to be pathogenic for turkeys.

TABLE 3

THE *IN VITRO* ANTIBIOTIC SENSITIVITY OF ISOLATES OF PPLO PROCURED FROM POULTS HATCHED FROM TURKEY HENS ON ANTIBIOTIC THERAPY AS COMPARED TO THE ORIGINAL CONTROL ISOLATE

Turkey group isolate	Antibiotic pretreatment*	Minimum inhibitory concentration (μg. /ml.)					
		Streptomycin	Dihydrostreptomycin	Tetracycline	Chlortetracycline	Oxytetracycline	Erythromycin
I P	None (poult control)	200.00	400.00	1.56	0.78	1.56	100.00
II P	Dihydrostreptomycin Tetracycline	100.00	100.00	12.50	3.13	3.13	100.00
III P	Tetracycline	200.00	400.00	200.00	50.00	50.00	100.00
IV P	Chlortetracycline	6.25	6.25	50.00	200.00	50.00	100.00
V P	Chlortetracycline	6.25	3.13	50.00	100.00	50.00	100.00
VI P	Oxytetracycline	200.00	400.00	100.00	100.00	100.00	100.00
VII P	Oxytetracycline	3.13	6.25	50.00	100.00	100.00	100.00
VIII P	Dihydrostreptomycin	200.00	400.00	1.56	0.78	1.56	100.00
IX P	Erythromycin	3.13	6.25	1.56	0.78	0.78	100.00
Original isolate	None	3.13	6.25	1.56	1.56	0.78	100.00

* See TABLE 1 for antibiotic treatment protocol.

The data presented in TABLE 4 indicate the MIC of antibiotic necessary to inhibit each isolate as tested against the specific antibiotic or antibiotics used to produce the isolate. Also indicated is the degree of resistance observed in each isolate. The data indicate that the degree of resistance to dihydrostreptomycin and tetracycline was lower when the drugs were used in combination than when they were administered singly (Groups III and VIII, TABLE 4). It is also interesting to note that cross resistance was observed among the "tetracycline" antibiotics. This degree of cross resistance is further indicated in TABLE 5.

TABLE 4

ANTIBIOTIC RESISTANCE INDUCED *IN VIVO* IN ADULT TURKEYS AND THEIR PROGENY BY PROLONGED ANTIBIOTIC THERAPY OF THE ADULTS

Turkey group	Antibiotic*	Results of *in vitro* sensitivity tests				
		Minimum inhibitory concentration (mg./ml.)				
		Isolate from adults after antibiotic treatment	Isolate from poults after antibiotic treatment	Original isolate before antibiotic treatment	Degree of resistance,† adult isolates	Degree of resistance,† poult isolates
II	Dihydrostreptomycin	50.00	100.00	6.25	8	16
	Tetracycline	6.25	12.50	1.56	4	8
III	Tetracycline	100.00	200.00	1.56	64	128
IV	Chlortetracycline	200.00	200.00	1.56	128	128
V	Chlortetracycline	200.00	100.00	1.56	128	64
VI	Oxytetracycline	100.00	100.00	0.78	128	128
VII	Oxytetracycline	100.00	100.00	0.78	128	128
VIII	Dihydrostreptomycin	400.00	400.00	6.25	64	64
IX	Erythromycin	100.00	100.00	100.00	1	1

* See TABLE 1 for treatment protocol of the turkey groups.

† Ratio of the MIC of the isolate of PPLO after antibiotic treatment to that of the original isolate before antibiotic treatment.

TABLE 5

ANTIBIOTIC CROSS RESISTANCE OF STRAINS OF PPLO RESISTANT TO TETRACYCLINE, CHLORTETRACYCLINE, OR OXYTETRACYCLINE

Turkey group	Antibiotic to which resistance was previously developed*	Tetracycline		Chlortetracycline		Oxytetracycline	
		MIC (mg./ml.)	Degree of resistance†	MIC (mg./ml.)	Degree of resistance†	MIC (mg./ml.)	Degree of resistance†
II	TC/DHS	6.25	4	3.13	2	1.56	2
III	TC	100.00	64	25.00	16	25.00	32
IV	CTC	50.00	32	200.00	128	50.00	64
V	CTC	50.00	32	200.00	128	50.00	64
VI	OTC	50.00	32	100.00	64	100.00	128
VII	OTC	50.00	32	100.00	64	100.00	128
Original isolate	None	1.56	—	1.56	—	0.78	—

* See TABLE 1 for treatment of the turkey groups with antibiotics, and abbreviations used.

† Ratio of the MIC of the isolate of PPLO after antibiotic treatment to that of the original isolate before antibiotic treatment.

Short-term antibiotic therapy experiments. TABLE 6 indicates the results of treatment of naturally infected flocks that had been treated with dihydrostreptomycin and in which the disease had reoccurred clinically after treatment. When these flocks were retreated with other antibiotics the disease was brought under control, except in the case of the dihydrostreptomycin-treated group.

Isolates of PPLO made previous to treatment and following treatment were tested for antibiotic resistance; as expected, they were found to be resistant to dihydrostreptomycin. It may be observed in TABLE 7 that this isolate, which is highly resistant to dihydrostreptomycin, was induced by a single injection of this antibiotic. The other isolates, when tested against the specific antibiotic used for treatment, were found to be as sensitive to the antibiotic as was the original strain. The dihydrostreptomycin-resistant strain, when inoculated into experimental birds, was highly pathogenic and rapidly produced clinical signs and a serologic response in the birds.

Table 6

Short-Term Antibiotic Therapy of PPLO-Infected Birds

Group	Drug pretreatment*	Treatment*	Effectiveness† (per cent)
A	DHS 50 mg./sinus	CM 50 mg./sinus	91.4
B	DHS 50 mg./sinus	EM 50 mg./sinus	89.6
C	DHS 50 mg./sinus	OTC 50 mg./sinus	84.5
D	DHS 50 mg./sinus	DHS 50 mg./sinus	36.6
E	DHS 50 mg./sinus	Saline control	3.2

* Key to abbreviations: DHS, dihydrostreptomycin sulfate; CM, carbomycin sulfate; OTC, oxytetracycline (experimental injectable); and EM, erythromycin (ethylsuccinate).

† Scoring was done 3 weeks postinoculation, with each group containing a minimum of 200 birds.

Table 7

The *in Vitro* Antibiotic Sensitivity of Isolates Obtained from Antibiotic-Treated Adult Turkeys Following Short-Term Therapy as Compared to the Original Isolate

Turkey group isolate	Antibiotic treatment*	Dihydrostreptomycin	Oxytetracycline	Erythromycin	Carbomycin
D	DHS 50 mg./sinus	800	<.39	3.12	<.39
Original	None	1.56	<.39	3.12	<.39

* See TABLE 6 for treatment protocol.

Conclusion

Antibiotic-resistant strains of PPLO were produced in adult turkeys following prolonged antibiotic therapy. The exception to the antibiotics tested was erythromycin. This agent, however, was not particularly sensitive to erythromycin before therapy. The antibiotic-resistant strains were egg-transmitted from the infected hens to their progeny. From experimental field data it was observed that a single injection of dihydrostreptomycin may induce PPLO strains that are resistant to this drug. Strains resistant to the tetracycline group of antibiotics apparently are produced only under the unusual circumstance of prolonged antibiotic therapy. Also, it is clear that erythromycin, carbomycin, and oxytetracycline are of value in the treatment of those outbreaks of infectious sinusitis in which a strain resistant to dihydrostreptomycin is involved.

Acknowledgments

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PLEUROPNEUMONIALIKE ORGANISMS: THEIR EFFECT ON CHICKEN CHORIOALLANTOIC MEMBRANE AND THEIR RESISTANCE TO ANTIBIOTICS

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In view of the possible relationships between pleuropneumonialike organisms (PPLO) and nongonococcal urethritis and because of the heavy increase of the latter in Germany (Marchionini and Röckl, 1957), England (Willcox, 1958), and in other countries, the dermatologist is interested particularly in three questions among the problems dealing with the pathology and microbiology of PPLO. (1) Are any cases of urethritis and vaginitis exclusively produced by PPLO? (2) Are there any PPLO strains having primary pathogenicity for humans; which are the conditions that may give them secondary pathogenicity? (3) Which is the optimal method of eliminating PPLO present in the human body?

Let us consider first the question of the relationship between the PPLO and nongonococcal urethritis. We studied 443 patients, consisting of 277 men, 129 women, and 37 children for the presence of PPLO in the genitourinary tract. Serum-agar plates were used for the cultivation of the organisms. The investigations conducted on men covered 115 healthy persons without urethritis and 162 patients having diseases of the genitourinary tract. TABLE 1 shows the frequency of PPLO determinations made in the individual groups. The percentage difference of positive PPLO determinations between healthy men and those with nongonococcal urethritis is not significant.

In women PPLO can be isolated from the genitourinary tract much more frequently than in men (TABLE 2).

Examinations were conducted also in children (TABLE 3).

These clinical studies yielded the following results of particular interest: 5 cases of nongonococcal urethritis were observed in the group of men from whom neither bacteria nor PPLO could be isolated. In another case of urethritis, however, a great number of PPLO were found without the presence of any bacteria. In 6 men PPLO could be demonstrated apart from *Trichomonas vaginalis*.

In 31 prostitutes we found *T. vaginalis* in 26 per cent, and PPLO in 74 per cent of the cases. Of the 117 women examined, 16.2 per cent showed the simultaneous presence of PPLO and *T. vaginalis* in the cervical and urethral secretion. Some women with gonorrhea also showed PPLO and *T. vaginalis* in the genital flora.

The relatively frequent occurrence of PPLO in the female genitourinary tract seems to indicate that these organisms are probably harmless saprophytes that perhaps may be classified among the regular flora of the genitourinary tract.

This opinion appears to be corroborated by the fact that PPLO, without causing any symptoms, occur in the healthy urethra of males, and that these organisms were found even in four healthy children. The high percentage of PPLO infections observed in prostitutes suggests that social and sexual-sanitary conditions may play a certain role in regard to the acquisition of PPLO. The risk of infection is likely to be increased by frequent change in sexual partners.

The question whether there are varieties of urethritis and vaginitis caused exclusively by PPLO could not be answered by our clinical investigations.

Let us now focus our attention on the second question concerning the pathogenicity of the PPLO. It is difficult to classify the PPLO into the system of microorganisms, since they pass through ultrafilters in the same way as do the viruses. On the other hand, in contrast to viruses, they can be cultured on cell-free nutrient media. They are thus able to multiply without requiring the chemical services of the host cells (Klieneberger-Nobel, 1938, 1954; Tulasne and Brisou, 1955; Edward and Freundt, 1956; Poetschke, 1952, 1954; and others).

TABLE 1

NUMBER OF POSITIVE PPLO CULTURES IN HEALTHY MEN AND IN PATIENTS WITH URETHRITIS AND PROSTATITIS

Number of men	Disease state	Positive PPLO cultures in a total of	PPLO determinations (per cent)
115	Healthy (without urethritis)	22	19.1
120	Nongonococcal urethritis	32	27
20	Chronic prostatitis	3	15
22	Gonorrhea	1	4.5

TABLE 2

NUMBER OF POSITIVE PPLO CULTURES IN HEALTHY WOMEN AND IN FEMALE PATIENTS WITH GONORRHEA

Number of women	Disease state	Positive PPLO cultures in a total of	PPLO determinations (per cent)
117	Healthy	73	62
12	Gonorrhea	9	75

Twenty-three (74 per cent) of 31 prostitutes harbored PPLO in the cervix and urethra.

TABLE 3

NUMBER OF POSITIVE PPLO CULTURES IN HEALTHY CHILDREN AND IN CHILDREN WITH NONGONOCOCCAL VULVOVAGINITIS

Number of children	Disease state	Positive PPLO cultures in a total of	PPLO determinations (per cent)
31 (both sexes)	Healthy	4	12.9
6 (girls)	Nongonococcal vulvovaginitis	1	17

Morphologically and microbiologically, the PPLO strains isolated by us corresponded to those that had been isolated from humans by numerous other authors (among others: Liebermeister, 1953*a* and *b*; Nicol and Edward, 1953; Kandler and his co-workers, 1954; Bartmann and Höpken, 1955; Peoples *et al.*, 1957). The majority of the organisms grown by us showed diameters ranging between 200 and 500 mμ; only a few were smaller or considerably larger. Following exposure to the vapors of heavy metals under an angle of 15°, the organisms, especially the smaller ones with a diameter up to 350 mμ, revealed a shape similar to snowballs. The vesicular nature is seen more clearly in the larger particles. A good survey of the structure of the PPLO colonies is furnished by electron-microscope photographs of ultrathin sections that may be prepared from the serum-agar nutrient media following osmium fixation and embedding in methacrylate by means of the ultramicrotome.

For a long time organisms showing this structural principle have been known to be heavily pathogenic for a number of animals. Considerable sensation was excited, however, when Dienes and Edsall in 1937 were, for the first time, able to isolate PPLO in pure culture from a bartholinian abscess of a laboratory technician who handled rats (for PPLO infection from laboratory animals see Nelson, 1958).

In order to study the host spectrum of the PPLO strains isolated from humans, we inoculated not only white mice and other rodents, but also egg cultures by means of the chorioallantoic technique. Following inoculation of the PPLO into the chorioallantoic membrane (CAM), only about 45 per cent of the inoculated eggs showed a demonstrable multiplication of organisms, and a more pronounced growth was observed only in 13 per cent. In order to be able to evaluate the multiplication rate of the PPLO in the CAM, serum-agar nutrient media were inoculated with small pieces of CAM, and the number of colonies developing was determined.

TABLE 4
DEGREE OF MULTIPLICATION OF PPLO IN EGG CULTURES INFECTED WITH PPLO EXCLUSIVELY AND WITH PPLO TOGETHER WITH ECTROMELIA VIRUS

	PPLO alone				PPLO and ectromelia			
Degree of multiplication of PPLO in the incubated egg	0	+	++	+++	0	+	++	+++
Number of egg cultures	42	24	—	10	6	3	1	33
Total	76				43			

For improving the conditions of multiplication of the PPLO in the CAM, we used a method that Mooser (1951) and Mooser and Joos (1952) had applied for the first time in mice. Analogously, egg cultures were infected simultaneously with PPLO and virus of ectromelia. A profuse propagation of PPLO was obtained at a much higher percentage. In about 86 per cent of the egg cultures seeded in this manner, PPLO multiplied, 77 per cent showing a higher degree of growth (TABLE 4). The growth promotion of PPLO in the incubated egg is not a specific property of the virus of ectromelia. Other types of viruses of the pock group, including variola vaccinia, neurovaccinia, and rabbit myxoma virus, show the same effect (for further particulars see Nasemann *et al.*, 1954; and Röckl *et al.*, 1954). The increase in the multiplication rate of the PPLO by a symbiosislike process offers little confirmation for a stronger, primarily existing virulence of the organisms.

In a number of men and women in whom we could demonstrate PPLO, we performed complement fixation reactions with the blood serum and a PPLO antigen. To date, we have not found any clue for the development of specific antibodies.

In agreement with Freundt (1956), we are of the opinion that there are more indications in favor of the fact that the PPLO occurring in the urethra and vagina of humans are, as a rule, harmless saprophytes. The fact, however, that humans harbor microorganisms that may produce serious diseases in animals is of interest at least from a heuristic point of view. A definite answer cannot be given as yet to the questions of whether there are any PPLO strains primarily pathogenic for humans or under what conditions saprophytic PPLO may acquire secondary pathogenicity.

Only the third question posed in this context, that is, the possibility of therapeutically influencing the PPLO, can be answered with certainty. Owing to the administration of Aureomycin, we succeeded in many cases in eliminating PPLO from the vaginal flora or from the male urethra. *In vitro* studies for determining resistance revealed that penicillin and several sulfa drugs do not exert an appreciable influence on the PPLO isolated by us. Streptomycin and Chloromycetin (chloramphenicol) show a good effect, whereas Aureomycin and other tetracyclines are very effective. Aureomycin produces a marked inhibition of multiplication at a level as low as 0.5 μg./cc., and a complete inhibition at a level of 5 to 10 μg./cc.

The resistance of PPLO to antibiotics was tested not only *in vitro*, but also *in vivo* by means of a total of 317 egg cultures. Various sulfonamides and antibiotics were added to the incubated eggs in rising concentrations. In agreement with the *in vitro* tests, the investigations showed that penicillin and some sulfa drugs are unable to inhibit the propagation of the organisms. However, Aureomycin and other tetracyclines exhibited a good effectiveness. At a level of 0.5 μg./gm. of egg, Aureomycin produced a marked inhibitory effect on the multiplication of the PPLO. Complete inhibition occurred at a level of 5 μg./gm. of egg and higher. In summarizing the results of our investigations, we emphasize the simple fact that, as in many other fields of modern medicine, our present knowledge of the pathogenetic role of PPLO has been surpassed greatly by recent advances in modern therapy.

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ANTIBIOTIC CONTROL OF AN EXPERIMENTAL *MYCOPLASMA GALLINARUM* (PPLO) INFECTION IN CHICKENS

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Introduction

Chronic respiratory disease (CRD) of chickens and infectious sinusitis of turkeys cause serious economic losses each year, although accurate figures on these losses are not available.

The work of Markham and Wong,[1] confirming the much earlier work of Nelson,[2,3] strongly indicated an etiological role for pleuropneumonialike organisms (PPLO) in both diseases, and the work of Van Roekel and Olesiuk,[4] among others, has confirmed that finding, although an ancillary role is claimed for Gram-negative bacteria[5] or a virus.[6] We have established a reproducible PPLO infection in chicks that we believe can be used to compare the activity of several antibiotics shown to be effective in the treatment of CRD of chickens and infectious sinusitis of turkeys.

Materials and Methods

One-day-old Hallcross sex-linked cockerels weighing 30 to 42 gm. were infected intra-abdominally with 1 ml. of a yolk-sac suspension of *Mycoplasma gallinarum* ASD* diluted 10^{-1} in brain-heart infusion broth (Difco). The suspension was prepared from embryos dying on the seventh day after infection with this strain that was obtained originally from E. Jungherr, University of Connecticut, Storrs, Conn., and has been passed by the yolk-sac route through chick embryos, weekly, for more than 3 years.

Control chicks were injected in the same manner with yolk-sac suspensions from uninfected embryos. Brooder heat was turned off on the seventh day after infection. This acted as a stress factor in birds of that age.

Each test group consisted of 20 chicks. In the experiments in which the birds were treated by drug diet, they were given the drug diet immediately after infection and for 3 weeks thereafter. When treatment was parenteral, the chicks were injected intra-abdominally with 1 ml. of a distilled water dilution of the antibiotic once daily, beginning the day after infection.

The controls were injected with 1 ml. of distilled water. All tests were terminated on the twenty-ninth day. Mean survival times were calculated on the birds that died. The criteria for estimating the effect of the infection and the activity of the antibiotic were: (1) mortality, (2) weight gains, and (3) appearance of, or increase of, specific agglutinins.

Deaths occurring during the first seven days were considered due to factors other than the *M. gallinarum* (PPLO) infection, such as trauma or toxicity. Later deaths were attributed to the infection. Each bird was weighed on the eighth, fifteenth, twenty-second, and twenty-ninth days after infection. The mean weight of the infected-untreated group was compared with the mean weight of each of the other groups by means of the t test to determine whether differences were significant. A reduced weight gain is characteristic of this infection but, since it is not specific for it, we obtained more specific information by means of the serum-agglutination test.

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* Air-sac disease. This strain has been shown to be of Type S-6 by Julius Fabricant, Cornell University, Ithaca, N.Y.

Serum samples taken from the birds at the beginning and at the end of the experiment were tested by the plate technique,[7] using a stained PPLO antigen supplied by F. S. Markham of the Viral and Rickettsial Research Section. A large percentage of positive sera from the infected groups, especially those untreated or treated with less than fully effective doses of active drugs, suggests a causal relationship between the PPLO infection and decreased weight gains.

TABLE 1

SURVIVAL DATA, MEAN BODY WEIGHT WITH STANDARD DEVIATION, T VALUES, AND NUMBER OF BIRDS WITH POSITIVE AGGLUTININ TITERS 29 DAYS AFTER INFECTION WITH *M. GALLINARUM* AND TREATMENT WITH CHLORTETRACYCLINE, SPIRAMYCIN, OR LEUKOMYCIN AT 400, 200, OR 100 PPM IN THE DIET

Antibiotic	Dose (ppm)	Survival* ratios	Survival percentages	Mean survival time (days)	Mean weight (gm.)	S.D.	t†	Agglutinin‡ ratios
Uninfected, untreated	none	18/18	100	—	421	37	10.2	0/18
Infected, untreated	none	13/20	65	24	165	98	—	6/6
Leukomycin	400	20/20	100	—	401	85	7.38	8/20
	200	19/20	95	29	406	90	7.13	15/17
	100	11/20	55	23	218	75	1.47	10/10
Spiramycin	400	20/20	100	—	439	35	11.5	4/20
	200	20/20	100	—	440	50	10.7	7/20
	100	19/20	95	24	379	80	6.73	11/19
Chlortetracycline	400	20/20	100	—	424	37	10.8	6/20
	200	18/20	90	16	401	72	7.76	7/18
	100	18/19	95	24	412	82	7.65	9/17

* Number of birds alive/total alive 7 days after infection.

† Values of 3.55 or higher represent significance at probabilities of less than 0.001.

‡ Number of birds with titers of $\geq 1{:}2$/total number tested.

We did not attempt to use a lesion score as a criterion of the effectiveness of the drugs because we found that rather severe lesions might be found in birds that had made normal weight gains and, conversely, that birds that had only slight lesions might not have made satisfactory weight gains.

The infecting organism was isolated many times from pectoral bursae, heart, synovial fluid, and the brain, but we did not attempt to use isolation of the infecting organism as a criterion of drug effectiveness because it is a relatively laborious procedure and because we felt that it had little relevance except to show that the birds had not been cleared completely of the infection.

Results

Comparison of chlortetracycline, spiramycin, and leukomycin at 400, 200, and 100 ppm in the feed. In this experiment 6 of 13 of the infected untreated chicks that survived had high agglutinin titers (TABLE 1). Blood samples could not be drawn from the others because of severe hemoconcentration.

All of the birds in the uninfected, untreated group were negative for PPLO gglutinins, even though 7 of 26 birds tested at the time of infection were posi- ve.

Nearly all of the birds treated with leukomycin at 200 or 100 ppm had high gglutinin titers, although the birds on 200 ppm had little mortality and made

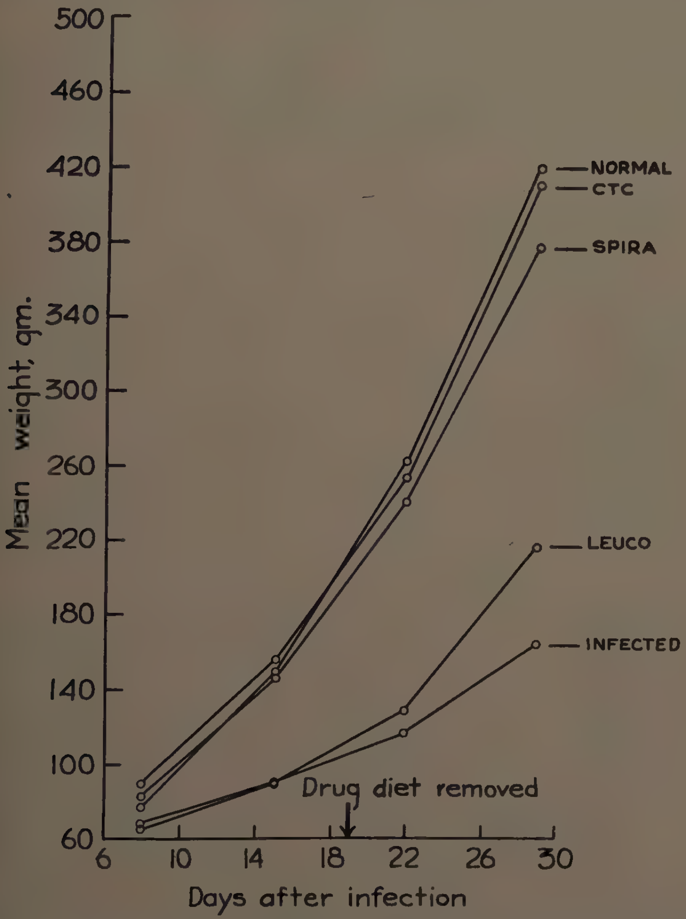

FIGURE 1. Comparison of leukomycin, spiramycin, and chlortetracycline in the feed at ppm used against an infection with PPLO in chicks.

od weight gains (TABLE 1). The group on 100 ppm had a high mortality, d their weight gain was not significantly better than that of the untreated, ected controls (FIGURE 1). Less than one half of the birds on spiramycin 400 and 200 ppm had high agglutinin titers. Slightly more than one half the group on 100 ppm had agglutinins, but all 3 groups made weight gains t were not significantly lower than those of the uninfected, untreated groups BLES 1 and 2 and FIGURE 1).

The results with chlortetracycline were much the same as those with spiramycin. Weight gains in every instance were as good as those of the uninfected controls. It should be noted that the groups of birds that made the best weight gains had the smallest standard deviation of weight, that is, there were few runts or culls in the group.

Comparison of chlortetracycline, spiramycin, and leukomycin at 200, 100, or 50 ppm in the feed. Since 400 and 200 ppm of each of the drugs had protected the birds from the infection, a second experiment was done in which the drugs were given at 200, 100, or 50 ppm in the feed. The infection was fairly severe. The infected, untreated group made only 56 per cent of the weight gain of the uninfected, untreated group and suffered a 45 per cent mortality (TABLES 3 and 4). Only 5 of the 36 birds surviving after treatment with leukomycin were negative for agglutinins. The groups treated with 100 or 50 ppm of leukomycin did not make significantly better weight gains than did the untreated, infected group. The birds on spiramycin at all 3 levels made as good weight gains as did the uninfected birds and suffered no mortality, but one half of them had high PPLO-agglutinin titers. Chlortetracycline at 200 ppm prevented mor-

TABLE 2

MEANS AND RANGES OF WEIGHTS AT EACH WEIGHING: FIRST EXPERIMENT WITH LEUKOMYCIN, SPIRAMYCIN, AND CHLORTETRACYCLINE IN FEED OF BIRDS INFECTED WITH *M. GALLINARUM*

Antibiotic treatment	Days after infection			
	8	15	22	29
	Mean weight (gm.)			
Uninfected, untreated	78	148	263	421
Infected, untreated	66	89	117	165
Leukomycin, 400 ppm	75	145	258	401
200 ppm	87	157	260	406
100 ppm	68	89	129	218
Spiramycin, 400 ppm	89	166	270	439
200 ppm	88	166	289	440
100 ppm	84	147	241	379
Chlortetracycline, 400 ppm	90	165	275	424
200 ppm	85	155	255	401
100 ppm	89	155	256	412
	Range of weights (gm.)			
Uninfected, untreated	61–88	125–173	225–312	365–501
Infected, untreated	55–83	68–125	81–219	75–361
Leukomycin, 400 ppm	59–98	91–188	119–316	142–507
200 ppm	67–107	82–199	91–352	98–515
100 ppm	55–81	59–117	66–191	111–322
Spiramycin, 400 ppm	73–105	130–191	213–303	357–488
200 ppm	68–105	118–205	209–351	329–535
100 ppm	70–97	87–183	81–314	174–487
Chlortetracycline, 400 ppm	74–107	118–188	200–305	317–473
200 ppm	65–107	108–195	103–299	141–466
100 ppm	78–103	87–197	90–344	151–529

Average weight/bird 1 day old was 36 or 37 gm.; 7/26 positive for specific agglutinins.

TABLE 3

SURVIVAL DATA, MEAN BODY WEIGHT WITH STANDARD DEVIATION, T VALUES, AND NUMBER OF BIRDS WITH POSITIVE AGGLUTININ TITERS 30 DAYS AFTER INFECTION WITH *M. GALLINARUM* AND TREATMENT WITH CHLORTETRACYCLINE, SPIRAMYCIN, OR LEUKOMYCIN AT 200, 100, OR 50 PPM IN THE DIET

Antibiotic	Dose (ppm)	Survival ratios	Survival per-centages	Mean survival time (days)	Mean weight (gm.)	S.D.	t*	Agglu-tinin† ratios
Uninfected, untreated	none	19/19	100	—	441	38	6.86	0/19
Infected, untreated	none	11/20	55	16	251	111	—	8/10
Leukomycin	200	19/20	95	23	381	76	3.81	15/19
	100	10/20	50	23	285	97	0.74	9/9
	50	9/20	45	16	274	115	0.45	7/8
Spiramycin	200	18/20	90	19	425	72	5.12	12/18
	100	19/20	95	21	446	44	6.86	9/19
	50	20/20	100	—	432	74	5.46	7/20
Chlortetracycline	200	19/20	95	30	420	58	5.52	4/19
	100	17/19	90	22	372	95	3.08	6/16
	50	16/19	84	18	348	61	2.93	7/16

* Values greater than 2.64 represent significance at a probability of less than 0.01.
† Number of birds with agglutinin titers of ≧1:2/total number tested.

TABLE 4

MEANS AND RANGES OF WEIGHTS AT EACH WEIGHING: SECOND EXPERIMENT WITH LEUKOMYCIN, SPIRAMYCIN, AND CHLORTETRACYCLINE IN FEED OF BIRDS INFECTED WITH *M. GALLINARUM*

Antibiotic treatment	Days after infection			
	9	16	23	30
	Mean weight (gm.)			
Uninfected, untreated	82	164	284	441
Infected, untreated	69	108	170	251
Leukomycin, 200 ppm	90	156	255	381
100 ppm	72	109	159	285
50 ppm	70	105	184	274
Spiramycin, 200 ppm	87	164	280	425
100 ppm	92	172	298	446
50 ppm	92	170	283	432
Chlortetracycline, 200 ppm	88	157	265	420
100 ppm	81	145	236	372
50 ppm	76	134	216	348
	Range of weights (gm.)			
Uninfected, untreated	68–97	130–194	238–338	382–529
Infected, untreated	52–84	64–152	82–255	94–394
Leukomycin, 200 ppm	66–110	71–200	172–322	259–485
100 ppm	54–92	57–182	51–305	122–458
50 ppm	55–97	56–173	101–285	112–423
Spiramycin, 200 ppm	52–100	85–190	148–332	225–514
100 ppm	66–104	70–201	215–339	341–506
50 ppm	71–117	106–217	148–363	216–535
Chlortetracycline, 200 ppm	70–105	92–204	93–348	304–528
100 ppm	62–101	102–189	101–320	108–486
50 ppm	52–87	89–157	89–270	205–435

Average weight/bird 1 day old: 36 to 39 gm.; 6/24 positive agglutinin.

tality and allowed weight gains that were as good as those of the uninfected group. At 100 ppm, growth was better than that of the infected, untreated group, but less than that of the uninfected controls. At a dose of 50 ppm, growth was depressed further and, at the end of the test, the mean weight of this group was significantly less than that of the uninfected birds. Several birds on the 2 lower doses died, and 17 of 51 birds in the 3 groups on chlortetracycline, alive at the end of the test, had high PPLO-agglutinin titers.

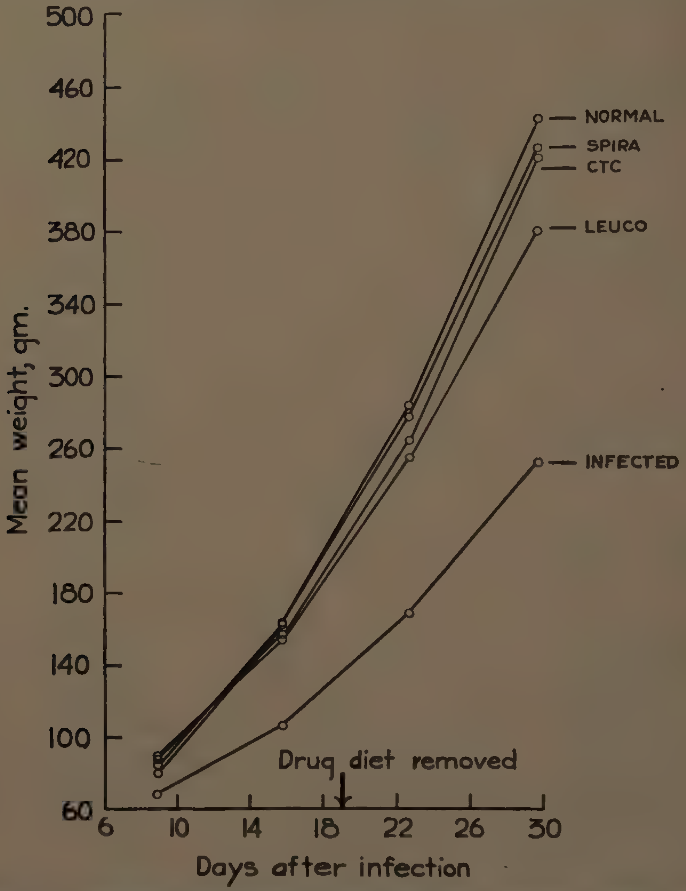

FIGURE 2. Comparison of leukomycin, spiramycin, and chlortetracycline in the feed at 200 ppm used against an infection with PPLO in chicks.

The growth curves for the birds on 200, 100, or 50 ppm of each drug are shown in FIGURES 2, 3, and 4.

Comparison of chlortetracycline, tetracycline, and erythromycin in the feed. Three experiments were done in which groups of PPLO-infected birds were treated with chlortetracycline at 200, 100, or 50 ppm, with tetracycline at 400, 200, or 100 ppm, or with erythromycin at 400, 200, or 100 ppm. The growth, mortality, and incidence of agglutinins for PPLO in the serum were compared with growth, mortality, and incidence of agglutinins in groups of infected, untreated and uninfected, untreated birds. The results of these tests are combined in TABLES 5 and 6 and in FIGURES 5 and 6.

The infection was fairly severe, as it caused a 33 per cent mortality and a depression of mean body weight of 109 gm. per bird, or 26 per cent, with an 80 per cent incidence of agglutinins for PPLO in the infected, untreated group.

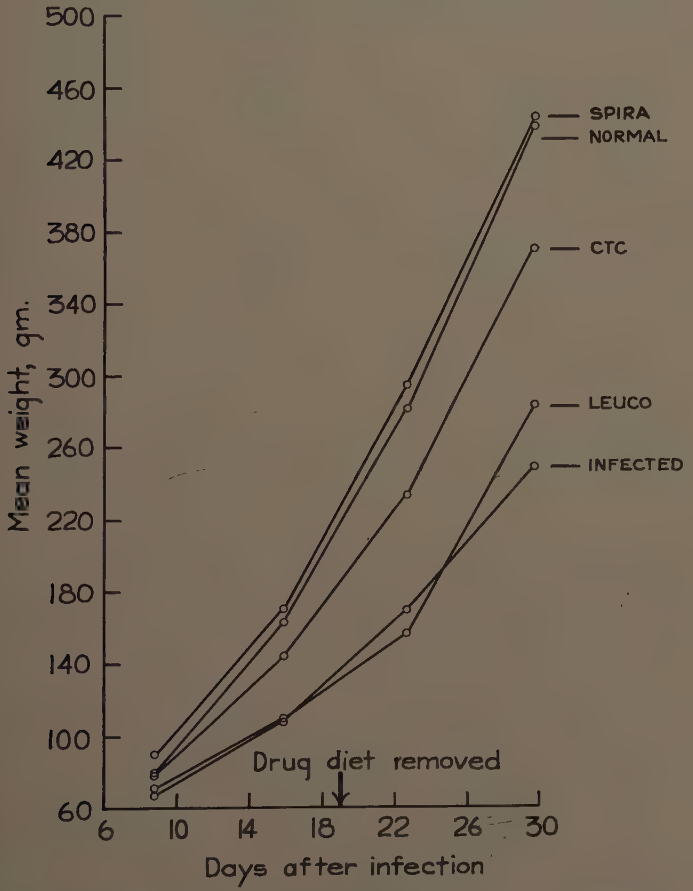

FIGURE 3. Comparison of leukomycin, spiramycin, and chlortetracycline in the feed at 100 ppm used against an infection with PPLO in chicks.

Chlortetracycline at 200 ppm almost completely controlled both mortality and depression of growth, but there still was a 60 per cent incidence of agglutinins for PPLO. At 100 ppm, however, there was considerable mortality, and the mean body weight of the birds was not significantly different from that of the infected, untreated group. The groups that received 50 ppm of chlortetracycline were not significantly different from the infected, untreated group in growth, mortality, or incidence of agglutinins.

There was considerable mortality at 400 ppm of tetracycline, but growth was significantly better than that of the infected, untreated group. The incidence of agglutinins was high. Tetracycline was ineffective at 200 or 100 ppm.

The results with erythromycin were unsatisfactory. The group on 200 ppm made a weight gain that was significantly better than that of the infected, untreated group, but the groups on 400 or 100 did not. All three groups had a high mortality. We believe that these results indicate that erythromycin is toxic to young birds at 400 ppm in the feed, and ineffective at 100 ppm. There may have been some effect at 200 ppm.

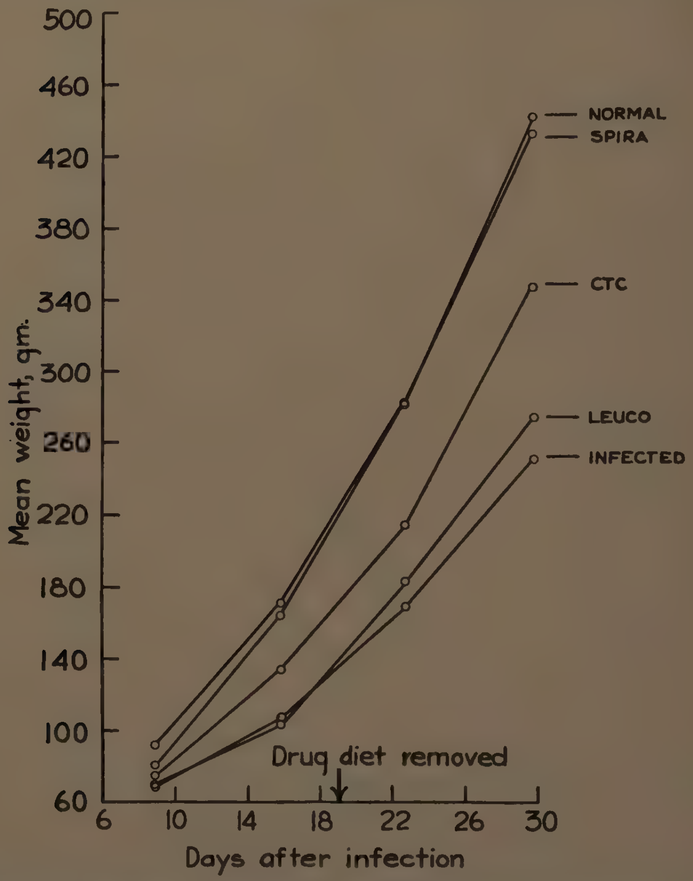

FIGURE 4. Comparison of leukomycin, spiramycin, and chlortetracycline in the feed at 50 ppm used against an infection with PPLO in chicks.

Growth curves are given for selected groups of birds in FIGURES 5 and 6.

Comparison of chlortetracycline, streptomycin, and spiramycin at 2.5, 1.25, or 0.63 mg./bird/day for 10 days, injected intra-abdominally. Because streptomycin is used widely for the control of infectious sinusitis, we decided to compare it with spiramycin and chlortetracycline. Since our objective was to compare the activity of the drugs, not to establish a method of control for use in the field, we decided to use multiple doses intra-abdominally.

Three experiments were performed in which each drug was given at 2.5, 1.25, or 0.63 mg./bird/day for 10 days. The results of these tests are combined in TABLES 7 and 8. All of the treated groups except the one on 0.63 mg. of spiramycin per bird made significantly better growth than the infected, untreated group, although the groups on 0.63 mg./bird of the other 2 drugs made poorer weight gains than the uninfected, untreated group. Only the group on 0.63 mg. of spiramycin suffered significant mortality.

TABLE 5

SURVIVAL DATA, MEAN BODY WEIGHT WITH STANDARD DEVIATION, T VALUES, AND NUMBER OF BIRDS WITH POSITIVE AGGLUTININ TITERS 29 DAYS AFTER INFECTION WITH *M. GALLINARUM* AND TREATMENT WITH CHLORTETRACYCLINE, TETRACYCLINE, AND ERYTHROMYCIN IN THE FEED

Antibiotic	Dose (ppm)	Survival ratios	Survival percentages	Mean survival time (days)	Mean weight (gm.)	S.D.	t*	Agglutinin† ratios
Uninfected, untreated	none	58/58	100	—	402	39	8.10	0/58
Infected, untreated	none	39/58	67	22	293	89	—	31/39
Chlortetracycline	200	52/57	91	27	383	93	4.55	31/51
	100	49/59	83	23	296	112	0.13	40/47
	50	36/60	60	22	295	115	0.08	25/31
Tetracycline	400	49/59	83	22	341	105	2.23	39/47
	200	38/56	68	23	313	120	0.79	30/36
	100	46/60	77	21	302	106	0.41	33/44
Erythromycin	400	33/58	57	19	337	108	1.84	20/33
	200	37/59	63	21	376	102	3.68	28/37
	100	44/59	75	22	329	120	1.50	27/41

* Values greater than 2.64 represent significance at a probability of less than 0.01.
† Number of birds with agglutinin titers of ≧1:2/total number tested.

TABLE 6

MEAN BODY WEIGHT OF BIRDS AT EACH WEIGHING: EXPERIMENTS WITH CHLORTETRACYCLINE, TETRACYCLINE, AND ERYTHROMYCIN IN THE FEED OF BIRDS INFECTED WITH *M. GALLINARUM*

Antibiotic treatment	Days after infection				
	0	8	15	22	29
	Mean body weight (gm.)				
Uninfected, untreated	36	76	143	257	402
Infected, untreated	36	70	104	171	293
Chlortetracycline, 200 ppm	37	83	144	236	383
100 ppm	36	78	124	190	296
50 ppm	36	74	110	168	295
Tetracycline, 400 ppm	36	81	132	214	341
200 ppm	36	79	118	183	313
100 ppm	36	76	118	186	302
Erythromycin, 400 ppm	36	78	118	204	337
200 ppm	36	82	127	203	376
100 ppm	37	82	127	208	329

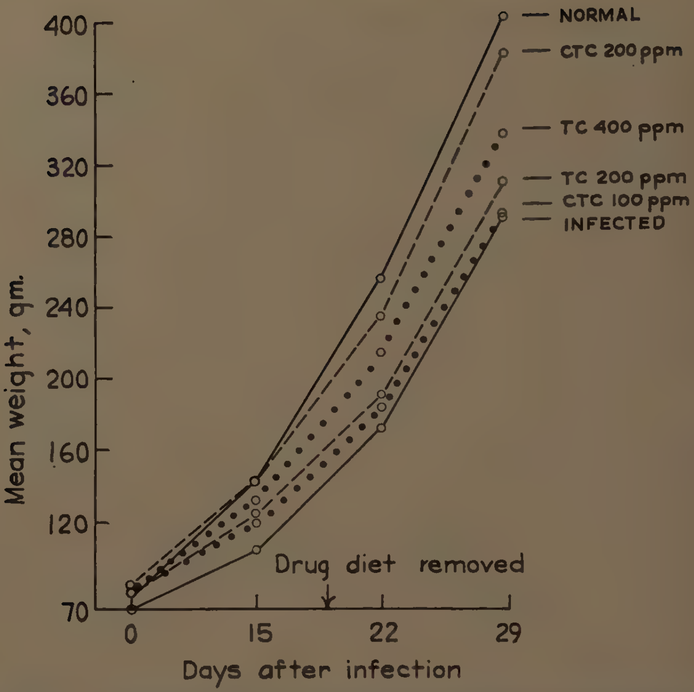

FIGURE 5. A comparison of chlortetracycline and tetracycline used against a PPLO infection in chicks.

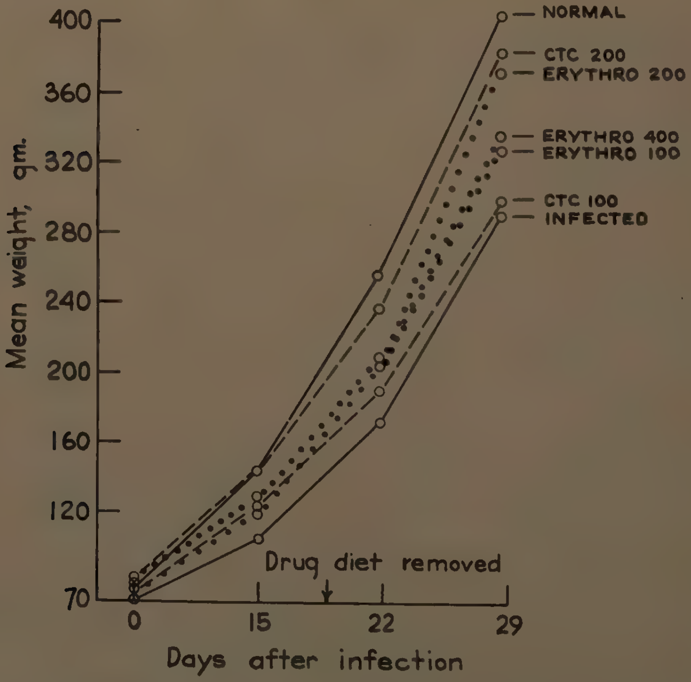

FIGURE 6. A comparison of chlortetracycline and erythromycin used against a PPL infection in chicks.

Comparison of chlortetracycline, streptomycin, and spiramycin at 2.5, 1.25, or 0.63 mg./bird/day for 5 days, injected intra-abdominally. A second series of experiments was performed in which the dose per bird of each drug was the same as in the previous series, but the number of doses given was reduced to 5. The survival data, mean body weights with standard deviation, and t values are given in TABLE 9. Only the group treated with 2.5 mg. of streptomycin per bird made significantly better weight gains than did the infected, untreated group. However, mortality was prevented almost completely except in the group receiving 0.63 mg. of spiramycin. It would seem that chlortetracycline

TABLE 7

SURVIVAL DATA, MEAN BODY WEIGHTS WITH STANDARD DEVIATION, AND T VALUES FOR EXPERIMENTS IN WHICH BIRDS INFECTED WITH *M. GALLINARUM* WERE GIVEN TEN INTRA-ABDOMINAL TREATMENTS WITH SPIRAMYCIN, STREPTOMYCIN, OR CHLORTETRACYCLINE

Antibiotic mg./bird	Survival ratios	Survival per-centages	Mean survival time (days)	Mean body weight (gm.)	S.D.	t*
Uninfected, untreated	52/54	96	15	404	65	5.70
Infected, untreated	35/55	64	20	304	99	—
Spiramycin, 2.5	55/58	95	10	407	56	8.78
1.25	54/57	95	11	371	73	3.57
0.63	47/56	84	24	326	107	0.95
Streptomycin, 2.5	64/64	100	—	389	51	5.67
1.25	60/60	100	—	370	60	3.97
0.63	59/60	98	25	343	86	2.01
Chlortetracycline, 2.5	60/61	98		372	53	4.24
1.25	58/59	98	28	365	58	3.77
0.63	51/57	90	27	343	73	2.11

* Values greater than 2.64 represent significance at a probability of less than 0.01.

TABLE 8

MEAN BODY WEIGHT AT 8, 15, 22, AND 29 DAYS AFTER INFECTION WITH *M. GALLINARUM* OF BIRDS GIVEN 10 INTRA-ABDOMINAL TREATMENTS WITH SPIRAMYCIN, STREPTOMYCIN, OR CHLORTETRACYCLINE

Antibiotic treatment	Days after infection			
	8	15	22	29
	Mean body weight (gm.)			
Uninfected, untreated	72	141	251	404
Infected, untreated	68	108	181	304
Spiramycin, 2.5 mg./bird	71	145	257	407
1.25 mg./bird	70	142	240	371
0.63 mg./bird	73	133	192	326
Streptomycin, 2.5 mg./bird	72	142	247	389
1.25 mg./bird	70	136	234	370
0.63 mg./bird	70	136	216	343
Chlortetracycline, 2.5 mg./bird	72	138	238	372
1.25 mg./bird	70	132	230	365
0.63 mg./bird	67	121	210	343

and spiramycin were less effective than streptomycin under these test conditions. The data on growth of the birds are given in TABLE 10.

TABLE 9

SURVIVAL DATA, MEAN BODY WEIGHTS WITH STANDARD DEVIATION, AND t VALUES FOR EXPERIMENTS IN WHICH BIRDS INFECTED WITH *M. GALLINARUM* WERE GIVEN FIVE INTRA-ABDOMINAL TREATMENTS WITH SPIRAMYCIN, STREPTOMYCIN, OR CHLORTETRACYCLINE

Antibiotic mg./bird	Survival ratios	Survival percentages	Mean survival time (days)	Mean body weight (gm.)	S.D.	t*
Uninfected, untreated	37/38	97	12	393	51	2.95
Infected, untreated	26/40	65	17	338	96	—
Spiramycin, 2.5	40/40	100	—	366	67	1.39
1.25	36/37	97	20	358	86	1.09
0.63	34/39	87	25	365	72	1.25
Streptomycin, 2.5	38/38	100	—	387	48	2.70
1.25	39/40	98	20	369	66	1.54
0.63	34/38	90	23	344	79	0.34
Chlortetracycline, 2.5	40/40	100	—	373	54	1.90
1.25	33/35	94	26	347	67	0.33
0.63	38/39	97	20	350	95	0.49

* Values greater than 2.66 represent significance at a probability of less than 0.01.

TABLE 10

MEAN BODY WEIGHT AT 8, 15, 22, AND 29 DAYS AFTER INFECTION WITH *M. GALLINARUM* OF BIRDS GIVEN 5 INTRA-ABDOMINAL TREATMENTS WITH SPIRAMYCIN, STREPTOMYCIN, OR CHLORTETRACYCLINE

Antibiotic treatment	Days after infection				
	0	8	15	22	29
	Mean body weight (gm.)				
Uninfected, untreated	37	70	141	247	393
Infected, untreated	36	64	119	209	338
Spiramycin, 2.5 mg./bird	36	67	138	233	366
1.25 mg./bird	36	70	139	230	358
0.63 mg./bird	37	72	137	226	365
Streptomycin, 2.5 mg./bird	36	69	137	237	387
1.25 mg./bird	35	67	133	229	369
0.63 mg./bird	36	68	132	218	344
Chlortetracycline, 2.5 mg./bird	35	69	130	227	373
1.25 mg./bird	36	68	133	217	347
0.63 mg./bird	36	72	138	231	350

Discussion

The use of antibiotics to combat CRD is very widespread, as shown by the large amount of literature on the subject. Our results may be compared with those of Van Roekel and Olesiuk,[4] who tested chlortetracycline, bacitracin, chloramphenicol, carbomycin, *p*-aminobenzoic acid, streptomycin, and oxytetracycline in turkeys or chickens experimentally infected with PPLO. These investigators used a lesion score as the criterion of activity, supplemented in some instances by data on weight gains. The results with carbomycin or oxytetracycline appeared to be better than those with chlortetracycline or streptomycin; the other drugs were clearly inactive. However, the data on infected, untreated and uninfected, untreated birds showed, and in fact the authors stated, that the infection from test to test was not uniform. The doses of chlortetracycline used were usually small and the period of treatment was usually 7 days. In one instance in which 360 gm. of chlortetracycline per ton of feed (400 ppm) was given for 14 days, the weight-gain data indicated that the drug was effective. In the later publication from the same laboratory[8] it was stated that of 10 products tested only 4 (chlortetracycline, oxytetracycline, carbomycin, and furazolidone) exerted any inhibitory effect on the infection. Erythromycin, tetracycline, dibenzylethylenediamine dipenicillin G, dihydrostreptomycin, streptomycin, and Pabakay (potassium *p*-aminobenzoate and procaine penicillin) were also tested. The evaluation of the activity of the drugs was based entirely on symptoms and lesions. We believe that there is not necessarily any correlation between severity of lesions, on the one hand, and weight gains and mortality, on the other, and that the latter are the more important criteria of activity.

Fahey and Crawley[9] also have published information on the effect of antibiotics on the clinical and serologic course of CRD. They concluded that the feeding of either chlortetracycline or oxytetracycline at 200 gm./ton of feed for 7 to 10 weeks failed to influence the clinical course of CRD. They did say that the antibiotics improved feed efficiency and reduced mortality. Also they demonstrated that feeding the antibiotic suppressed the appearance of hemagglutination-inhibition (HI) antibodies; however, the PPLO still could be isolated from chickens that had received the drug for 7 to 10 weeks continuously, even though they were negative to the HI test.

Price *et al.*[5] showed that oxytetracycline at 500 gm./ton of feed suppressed mortality and maintained a normal weight gain in birds infected with PPLO and then challenged with bronchitis virus. Penicillin at the same doses was ineffective.

Duerre and Carlson[10] reported that birds infected with PPLO and given chlortetracycline at 50 mg./pound of feed (100 ppm) made better weight gains than infected, untreated birds or uninfected, untreated birds, but not as good gains as uninfected birds treated with chlortetracycline. White-Stevens and Zeibel[11] fed chlortetracycline to broilers infected with PPLO. They reported that 50 or 100 gm. of chlortetracycline per ton of feed (50 or 100 ppm) maintained growth efficiency of broilers with a subclinical infection if the drug was fed continuously, but that 400 gm./ton or 250 mg./gallon of drinking water was required to maintain or restore growth efficiency when a clinical outbreak occurred, as happened when the birds became severely chilled.

Hamdy *et al.*[12] treated 20 large turkeys with 100 mg. of erythromycin per bird and reported that 18 recovered from clinical infectious sinusitis. The only other reference to the use of erythromycin against PPLO in poultry that we have seen is that of Adler *et al.*,[13] who treated the foundation breeding stock of 2 hatcheries with 25 mg./kg. of a mixture of dihydrostreptomycin and streptomycin per bird, supplemented with 0.4 gm. of erythromycin stearate per gallon of drinking water. Adler claimed to have prevented the egg transmission of PPLO by this method.

Conclusions

We believe that the following conclusions are warranted by the experiments just presented.

Tetracycline at 400 ppm, chlortetracycline or leukomycin at 200 ppm, or spiramycin at 50 ppm in the feed controlled rather severe experimental infections with PPLO. Erythromycin in young birds was unsatisfactory, as no dose gave normal growth or prevented mortality.

Streptomycin, chlortetracycline, and spiramycin at 0.63 mg./bird injected intra-abdominally once daily for 10 days controlled the infection but, when the number of doses was reduced to 5, only the 2.5-mg. dose of streptomycin was effective.

Summary

Spiramycin at 400 ppm down to 50 ppm in the feed maintained normal weight gains and prevented mortality in birds infected with *M. gallinarum.* A large proportion of the birds developed agglutinins for PPLO, although there were fewer positive at 400 or 200 ppm than at the lower doses.

Chlortetracycline at the same doses in the same tests at 400, 200, and 100 ppm gave good protection from mortality and gave weight gains as good as those of the uninfected group, but at 50 ppm several birds died and the weight gain, while significantly better than that of the infected, untreated birds, was not as good as that of the uninfected group. The agglutinin response was similar to that for spiramycin. In a second series of tests, in which tetracycline and erythromycin were compared to chlortetracycline, 100 ppm was less effective than in the first series, but 200 ppm was fully effective.

Leukomycin at 200 ppm maintained normal weight gains and prevented mortality, but failed to do so at 100 ppm and showed no effect at 50 ppm.

Tetracycline at 400 ppm was almost as good in controlling the infection as 200 ppm of chlortetracycline, but at 200 ppm tetracycline was ineffective.

The results with erythromycin were erratic, but 400 ppm appeared to be toxic; 200 ppm was almost as good as 200 ppm of chlortetracycline in maintaining growth, but did not prevent mortality; while 100 ppm failed to maintain growth but decreased mortality.

Spiramycin given intraperitoneally at a dose of 2.5 or 1.25 mg./bird/day for 10 days gave significantly better weight gains than were made by the untreated, infected birds, but 0.63 mg./bird/day did not permit growth significantly better than in untreated, infected birds, and a significant amount of mortality occurred. When the doses were the same but the number of treatments was reduced to 5, no dose tried gave a weight gain significantly better than that of the infected controls.

Chlortetracycline at 2.5, 1.25, or 0.63 mg./bird/day for 10 days gave significantly better growth than the untreated, infected birds and prevented mortality;

however, when the number of doses was reduced to 5, weight gains were not better than those of the untreated, infected birds, but mortality was prevented.

Streptomycin at 2.5, 1.25, or 0.63 mg./bird/day for 10 days prevented mortality and permitted growth significantly better than that of the untreated, infected controls. When the number of treatments was reduced to 5, only the 2.5 mg./bird/day/dose allowed weight gains better than those of the infected, untreated controls, but all three doses prevented mortality.

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DISCUSSION OF PART V

HARRY E. MORTON (*University of Pennsylvania, Philadelphia, Pa.*): I propose first to direct a few questions to Edward. It is a great problem to do serologic testing when PPLO cannot be made to grow in liquid media or if they cannot be grown in sufficient quantities to produce an antigen. Can the sensitivity of your growth inhibition test be used for serologic indentification of strains of PPLO that will grow only on a solid medium? Would this be a reliable method, and how would it compare with the complement fixation and hemagglutination reactions?

D. G. FF. EDWARD (*The Wellcome Research Laboratories, Beckenham, Kent, England*): I use this test more and more for serologic investigations because it eliminates the difficulty in preparing antigen, although it has the disadvantage of being more expensive with serum. There is much still to be learned about the test, which has been used only to examine a few species of the group. It was used extensively by me for human genital strains from which it is notoriously difficult to obtain good agglutinating suspensions. With the inhibition of growth test more than 90 strains were identified serologically with no technical difficulty.

In the serologic identification of strains the agglutination test has the disadvantage that there are minor differences between strains, so that one species-specific antiserum may not agglutinate all strains. For *Mycoplasma hominis* the inhibition of growth test had the advantage that it was less specific, and one serum inhibited the growth of all strains of the species. Subsequent experience has shown that this may not be true for every species, and there is a suggestion that the inhibition of growth test may sometimes be more specific than the agglutination test.

In preparing antisera in rabbits, growth-inhibiting antibodies do not develop at the same time as do the agglutinating antibodies. Sometimes they appear first; more often the rabbit has to be inoculated repeatedly until they develop. I should be interested to learn whether inhibiting antibodies can be demonstrated in animals recovering from natural infection.

I mention a general point on the preparation of antisera in rabbits. I notice that many workers prepare their antigens for inoculating rabbits in media enriched with horse serum. I consider that there is a great danger in doing this, owing to the risk of producing antibodies to horse serum. Before starting this type of work I consulted serologists and they told me that the results would always be open to criticism if horse serum were used. I have, therefore, always enriched my media with rabbit serum. Originally, with strains that grew only poorly in rabbit serum, I spent weeks and months adapting them to grow in rabbit serum media. Eventually I discovered that these strains would grow equally well in a rabbit serum medium as they did in a horse serum medium if cholesterol was added. As it is easy to grow a strain in a medium from which horse serum is eliminated, I strongly advocate that this should be done in order to avoid the criticism that the results may be vitiated by the presence of antibodies to horse serum.

In regard to the papers on tissue culture, I can report that I examined Collier's strain from the HeLa line using the growth-inhibition test. I identified it as *M. hominis*, Type 1. Subsequently I have isolated organisms of the pleuropneumonia group from cell lines maintained in my own laboratory. The organism from one cell line, originating in my laboratory, was identified as *M. gallisepticum*; it is possible that, on some occasion during the maintenance of this cell line, chick embryo extract had been added to the medium. The other two cell lines found to be contaminated had been obtained from other laboratories, one being HEp 2 line. Both were contaminated by a similar organism that it was impossible to identify.

MORTON: I wonder if Moore has any evidence as to which is the best species of animal to use for producing agglutinating serum. Are chickens any better than rabbits or other animals? Also, are the *Escherichia coli* strains that appear to exert a synergistic action in turkeys and chickens similar to the human strains of *E. coli*.

R. W. MOORE (*Agriculture and Mechanical College of Texas, College Station, Texas*): In answer to the first question concerning the best animal to use in making antisera, I believe that the turkey is the animal of choice for the avian strains because a natural infection is obtained in this case, and natural infection produces higher antibody titers. Usually a pathogenic strain in a turkey will give at least a 1:64 titer in the plate test; in the HI test, the titer is extremely high, more than 1:2560. Because I have a known PPLO-free strain of turkeys, I am using the turkey in diagnostic work. I inoculate a turkey with the PPLO exudate, or the exudate from the chicken or turkey and, within 6 days, an HI titer is obtained or, after about 8 days, a titer is obtained in the plate test. This added increase in titer occurs soon enough to permit the disease due to an avian strain to be diagnosed.

I lost that strain of *E. coli* before I could do very much with it.

MORTON: As a worker in the veterinary field Crawley has a good diagnostic test in the form of the hemagglutination test for detecting the presence of antibodies in poultry or for identifying strains that have been isolated. It would be very helpful if a similar test existed in the field of human medicine. Can Crawley tell us why human strains do not agglutinate red blood cells as do avian strains?

QUESTION: Does Crawley know if any cognizance is now being taken of the CRD virus in the control of chronic respiratory disease in Canada?

J. F. CRAWLEY (*University of Toronto, Toronto, Canada*): We still know nothing about the CRD virus. This virus has been isolated from turkeys several times and is, I believe, a cause of a distinct respiratory disease in the turkey. In chickens I have never encountered it by itself, as it always has been present in association with PPLO.

In answer to Morton's question concerning the use of an HI test for other PPLO: I do not know anything about that, but it is my impression that the pathogenic avian PPLO are perhaps the only ones that cause hemagglutination.

C. R. AMIES (*University of Alberta, Edmonton, Canada*): I obtained my knowledge of this subject from experience with L forms derived from a *Hemophilus* from the human genital tract. The hemagglutination test can be used for this L organism of *H. vaginalis*. The technique is simple.

MORTON: What evidence does Cover have of the presence of antibacterial antibodies if they are destroyed in 20 min. at 56° C.? I thought it was a criterion of a real antibody that it would survive 20 or 30 min. at 56° C., or even higher, as Coriell mentioned for his inactivation.

Morris S. Cover (*University of Delaware, Newark, Del.*): I also was slightly disturbed at the destruction of the agglutinating ability of a serum when heated at 56° C., because I was under the impression that when heated at this temperature the antibodies should be stable for at least 30 min. There is some evidence in the literature that not all antibodies are stable for this length of time at 56° C. Therefore, my results do not preclude the possibility that this is an antigen-antibody reaction.

Morton: Does Coriell know what is the best species of serum to use for the immune serum in the complement fixation test? Many people who employ this test use immune sera derived from a variety of species of animals.

You stressed the use of Terramycin in quantities sufficient to make the tissue cells sick in order to free the cells of their PPLO contaminants. Arthur Brown at the National Academy of Sciences, Washington, D.C., stated that his group of workers was successful in freeing tissue-cell lines by the use of Aureomycin. They used a high concentration of Aureomycin, made the cells sick, and then fed the tissue-cell lines to reduce the concentration of Aureomycin. It seemed necessary to make the tissue cells sick with the antibiotic before they were freed of the PPLO contaminants. Will that stress affect the properties of the tissue cells?

Lewis L. Coriell (*South Jersey Medical Research Foundation, Camden, N.J.*): The choice of the best animal sera to use for complement fixation tests is controversial. There are several factors that bear on this. For years virologists have used the rabbit because it produces good antibodies, but most workers no longer use the rabbit because pooled rabbit sera frequently fix complement. There seem to be isoantibodies, or some other difference in individual rabbits, to account for antibody in one rabbit's serum for the serum from another rabbit. Nonspecific fixation occurs in such pooled rabbit serum. Guinea pig serum does not do this as much; therefore the guinea pig is the favorite animal now. The use of chickens is successful for viral and bacterial antigens, and they have been used widely.

Bearing all of this in mind, I still choose the rabbit because it is the only available animal from which PPLO have not been isolated. This is important for people who work with avian PPLO. The turkey and the chicken will produce good antibodies, and they can be infected with PPLO. However, between the time they are injected and the time they are bled a month or two later to obtain the immune serum, these fowls may pick up another strain of PPLO from their environment. Thus the antibody obtained may not be highly specific. This is one of the problems to be guarded against and one of the reasons for selecting an animal that cannot be naturally infected, bearing in mind that it may present other problems. All of these possible difficulties must be controlled rigidly, and efforts must be made to avoid active infections.

People using guinea pigs, for example, in making antisera against the adenoviruses learned that the guinea pigs acquire natural infections with adenoviruses. This invalidated some early serologic work.

EMMY KLIENEBERGER-NOBEL (*The Lister Institute of Preventive Medicine, London, England*): May I ask Coriell why the HeLa cell cultures in various laboratories, all derived from one source, could not be infected with a human genital strain at the source? I also have examined a number of HeLa cell cultures with the result that they contain human genital strains. Would this not be a very simple explanation, as these PPLO are carried very easily. PPLO may be suppressed by the antibiotic. However, under certain conditions they may become more numerous.

JOHN T. SHARP (*Harvard Medical School, Boston, Mass.*): Has Coriell determined the *in vitro* sensitivity of the PPLO that he has obtained, and has he attempted treatment with combinations of antibiotics to which the organisms are sensitive? I think that may be of some practical importance, and it may give us a clue as to whether the organisms reside intracellularly or merely on the cells in these tissue cultures.

CORIELL: I have not done extensive work on the antibiotic sensitivity of these PPLO. I reviewed the literature and found that the wide-spectrum antibiotics, Aureomycin and Terramycin, seem to be the most effective—next to streptomycin, of course. My tissue cultures always contain 100 μg. of streptomycin per milliliter, so I did not test that antibiotic. PPLO obviously are resistant to that antibiotic. I have not tried combinations of antibiotics. This might be better, but I think that the process of treating PPLO with a sublethal dose of broad-spectrum antibiotic may have greater merit. This should be done at the time the tissue cells are off the glass, so that the cells will come in greater contact with the antibiotic. Perhaps this greater penetration of the antibiotic will eliminate the organisms from the tissue cells. I am immunizing, along the lines suggested by Edward, a horse for the purpose of using that immune serum as the source of serum in culture medium for tissue cultures. This may prove to be the simplest way to eliminate these organisms.

In response to the question of whether the HeLa line was infected at its source: I do not know the origin of the PPLO in the HeLa cultures or in any of the tissue cell lines. More information may show the source of the contaminant. However, I doubt whether we shall ever know this. The important point is that PPLO are in the tissue cell cultures, and the researcher should take whatever precautions and make whatever studies are necessary to determine how PPLO affects the system being studied.

GEORGE O. GEY (*Johns Hopkins Hospital, Baltimore, Md.*): More than 25 years ago I encountered an organism in tissue culture that none of the bacteriologists, except those who were also tissue culturists, could grow. One researcher attempted to prove that it was a rickettsial organism. Working with slides I was able to find colonies that then were the size of cells; these had been observed for a long time. When I placed a micromanipulator next to the cells, I was convinced that I had a microorganism that the bacteriologists could not grow.

In examining cells at very high magnifications, we see many contaminants. Recently I contaminated some specimens that I wanted to evaluate in special-phase microscopy. I contaminated these cell cultures with several organisms. These were HeLa cultures, but I had the choice of forty strains. These cells quickly ate up all the organisms, and within about four days the organisms were all gone. The problem of tissue cells destroying what might be considered an ordinary contaminant in the environment must be considered. Many of these organisms can be found in continuous cultures, and I should not consider any of our cells to be free.

The factor of quantity must now be considered. If in a metabolic study it is thought that an acquired enzyme or a new enzymatic activity has been discovered, the possibility that a microbe may be carrying on a nice carbohydrate utilization should be considered.

T. McP. BROWN (*George Washington University Hospital, Washington, D.C.*): Am I correct in assuming that, prior to the application of the techniques of Klieneberger-Nobel and Dienes, Gey did not know with what organism he was working?

GEY: I did not know.

BROWN: Did you know that these organisms were PPLO?

GEY: No.

COMMENT: May I say a few words about comparing the English strain of avian PPLO with some of the United States strains previously mentioned? I have isolated a number of PPLO from chickens. At first all PPLO that I found proved to be nonpathogenic when put into chickens. Later, I was able to improve the medium and to grow the pathogenic PPLO. The pathogenic PPLO were similar to Nelson's coccobacilliform bodies in culture.

If a culture is made on soft media a nonpathogenic PPLO grows. This growth appears in about 24 hours and, in smears, rings and bipolar bodies are found characteristic of the PPLO.

Three or four days later another type of colony is found to be growing. On examination of a single colony of this or a culture in broth, the coccobacilliform-body type of growth is found.

If this pathogenic organism, which resembles a coccobacilliform body, is put into a turkey sinus or into a chicken, a coryza or tracheitis is produced.

Thus it may be concluded that there are two types of PPLO in chickens, if Nelson's coccobacilliform bodies are called PPLO. One type, which has the morphology of coccobacilliform bodies, is pathogenic. The other type, which can be found in many diseases, is more pleomorphic.

During the past two years I have compared a number of my strains isolated from different sources with some of the strains isolated in the United States from subjects of CRD. I stress the importance of examining the medium more closely to determine whether different organisms can be grown. I believe that we agree that Nelson's coccobacilliform bodies are the cause of CRD. We regard the coccobacilliform bodies as pathogenic PPLO.

MORTON: Has Osborn any idea of how much antibiotic is contained in the chick embryo when the eggs are obtained from treated flocks? After the egg has been incubated and a usable embryo obtained, how much antibiotic may be remaining in that embryo?

O. H. OSBORN (*University of Minnesota, St. Paul, Minn.*): I do not have the answer to that question. At the time that I conducted the antibiotic sensitivity studies, I wanted to test sensitivity on the egg, but I did not know which bacterial organism to select as a test organism. Instead, I collected these eggs from each group and inoculated the original strain back into the eggs, and I tested antibiotic sensitivity on the original PPLO strain. At least from these experiments, I may say that the level would be below 1.56 μg. of antibiotic per milliliter.

The level of antibiotic transferred to the egg probably would be due to the antibiotic fed to the individual bird.

MORTON: We can discuss endlessly the relationship of PPLO to disease in man and perhaps not come to any definite conclusions. However, in glancing over the literature on the isolation of PPLO from the genitourinary tract of women, I found that there were 9 workers who have reported on a total of 682 women, of which 18 per cent were positive for PPLO. In contrast, 5 workers reported on studies of 583 clinic patients, 45 per cent of which were positive for PPLO. Many researchers have found that if there is pathology in the genitourinary tract, the possibility of isolating PPLO is much greater. Trying to relate PPLO to disease is very difficult. However, when PPLO are isolated in pure culture from the genitourinary tract in which there is pathology, and antibiotics are given, and when, in 1 to 3 days the PPLO disappear and the clinical symptoms begin to subside, this is good circumstantial evidence that PPLO were causing the pathology. Of course, it cannot be known if there is a virus lurking there that might have acted synergistically. Perhaps Paul Smith will describe one or two of the worst patients whom we studied and in whom we found pure cultures of PPLO.

PAUL F. SMITH (*University of Pennsylvania, Philadelphia, Pa.*): We had three cases in which there were acute symptoms of prostatitis or urethritis and conjunctivitis and, in one, a very typical arthritis. All of these individuals yielded PPLO. This was before Terramycin had come into use. The patients were treated with streptomycin, and the organisms and the symptoms disappeared. In none of these instances were we able to say that the PPLO caused the disease, although PPLO were isolated in pure culture. This is, of course, no more than circumstantial evidence.

T. NASEMANN (*University of Munich, Munich, Germany*): Some strains are primarily pathogenic while others are not. Because the antibiotics cure the patient and eliminate the PPLO it cannot be concluded that the PPLO were the cause of the trouble. Antibiotics have a wider activity.

MORTON: What does Kiser think about the diffusibility of spiramycin into mammalian cells?

JACKSON S. KISER (*American Cyanamid Company, Pearl River, N.Y.*): I assume that you are thinking of the possibility of using spiramycin in clearing tissue cultures of PPLO. I found that spiramycin was the most effective drug when given in the feed. It was also the most effective drug in some chick embryo experiments that I conducted. I do not know whether it diffuses into the tissue cells. It is highly effective in the chick embryos, and it is highly effective *in vitro* against the PPLO and should be tested in this respect.

SHARP: There are several points in Shepard's paper on human pathology that interest me. The problem of host-parasite relationship is curious because these are usually saprophytic organisms. In certain pathological conditions there arises the question whether the organisms are pathogens. Has something happened to the host to make it more susceptible or are there different strains? It would be interesting to know whether any serologic, morphologic, or biochemical studies have been done to indicate whether the strains isolated from disease situations are different from those isolated from healthy individuals.

Brown: In regard to the difference between animal and human strains, it has been found that erythromycin is not very effective against human strains. Apparently, it is effective against some of the animal strains. I have been working with chlortetracycline and have found that it is not at all effective or very poorly effective against one animal strain, L4, in which I have been particularly interested, whereas it is very effective against a number of human strains.

I once thought that perhaps some of the supposed saprophytes operate to produce their pathogenic effect, if such there be, by virtual sensitization of the surrounding tissue. Thus intracellular parasitism might actually be saprophytic or, certainly, nonpathogenic, and become pathogenic when such organisms are released into the surrounding tissue where antibody level perhaps had been developed.

As little as 10 mg. of Terramycin temporarily removes PPLO from prostatic cultures. The organisms will return in time, but 10 mg. actually will eliminate some strains. If phenylbutasone is given at the same time, tenfold the amount of Terramycin is required to have an effect on elimination of these organisms.

As phenylbutasone has no effect on PPLO, it might be thought that possibly a substance is released by the Terramycin, perhaps through destruction of a few organisms, and that those organisms release their antigens to react with tissue antibody, which is then effective in suppressing the growth of the organism. When that might be neutralized by an anti-inflammatory substance such as phenylbutasone, a greater amount of Terramycin therefore might be necessary. I have carried out that process in Reiter's disease. I have not been impressed with the results of antibiotics used against Reiter's disease, but phenylbutasone helped my patients. This suggests that perhaps a part of the pathogenesis is the tissue-hypersensitive mechanism.

Julius Fabricant (*Cornell University, Ithaca, N.Y.*): What relationship do these organisms have to the lesions in human beings? There is another possibility that the presence of lesions changes the biochemical environment sufficiently to allow these organisms to grow better. This is what happens in chickens with PPLO infection. A much better environment for the growth of certain secondary bacterial organisms is produced. The same type of mechanism may be operating here.

Question: Is resistance to penicillin associated with penicillinase production?

Morton: The PPLO are said to be the most penicillin-resistant organisms that do not produce penicillinase.

Part VI. Human Pathology

PATHOGENICITY AND IMMUNOLOGY OF ORGANISMS OF THE PLEUROPNEUMONIA GROUP

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It is likely that all pleuropneumonialike organisms (PPLO) are closely related. All have similar morphology and no rigid cell wall, and they occur as very small filterable elements and as bigger elements with a great deal of pleomorphism. Their colony form is characteristic, and they are more or less exacting in their nutritional requirements. They also have certain similarities in their pathogenicity. Therefore, the study of one disease caused by a particular type or species of PPLO can give us useful information for the elucidation of the etiology of disease caused by other species of PPLO (Edward, 1954; Klieneberger-Nobel, 1954).

Agalactia of sheep and goats, for example, is a disease of the mammary glands that breaks out in the lambing season. It affects lactating females with a purulent secretion appearing instead of the milk. The animals may have taken up the organisms many months previously while grazing in fields contaminated by an infected herd. Agalactia is usually a disease of the females only, but a systemic infection may occur and may affect the males also. This generalized disease manifests itself by swellings of the joints with a purulent synovial fluid and inflammation of the eyes so severe that the animals often lose their sight. The infection may lie latent in the animals for months, producing no symptoms, but it breaks out under stress and can develop into a severe systemic disease. The stress of the lambing is an additional factor necessary to precipitate the development of the disease in animals with latent infection.

An animal disease I have studied extensively for twenty-five years is the pulmonary disease of laboratory and wild rats, usually referred to as bronchiectasis or bronchopneumonia. D. Steabben and I (Klieneberger and Steabben, 1937) found that every rat of the Lister Institute breeding stock developed the disease when aging; these old rats were chronically sick. Healthy young rats, 2 months of age, after operations carried out for various purposes, sometimes became acutely ill with hepatization of the pulmonary lobes, glairy exudate, and large lung abscesses containing caseous pus. PPLO were cultivated in abundance on our special media from the lung lesions of both chronically and acutely diseased rats. In our 1937 report we remarked, "It is justifiable to say . . . that every rat in the stock . . . is predisposed to this infection." In later studies (Klieneberger and Steabben, 1940) we showed that the percentage of rats with PPLO in their lungs and the percentage of rats with lung lesions rose steeply with progressing age; the organisms were present before macroscopical lesions became manifest (TABLE 1). However, we could not fulfill Koch's third postulate, for we never succeeded in producing the pulmonary disease in healthy young rats by inoculation of the culture.

In any event a positive result would have meant nothing, because all the animals, as we later realized, probably had (in the uninjected state) latent infections of the organism.

In 1954 K.-K. Cheng showed that by ligating a bronchus of the rat he could invariably induce within one week a severe bronchiectasis in a young healthy animal 6 to 8 weeks of age. Subsequently, K.-K. Cheng and I (E. Klieneberger-Nobel and K.-K. Cheng, 1955) found in an investigation that on the first postoperative day no PPLO could be grown from the lungs, but that after 2 days the organisms could be grown from the lungs and that, after 4 days, they could be grown in abundance (TABLE 2). At 4 days, bronchiectasis was detectable and, after 12 days, it was pronounced, and abscesses had developed. PPLO must have been present in every rat at the time of operation, even when the rats were only 6 weeks old. We do not know whether the latent infection is transferred congenitally from the mother or during the suckling period. It appears in this case that the necessary precipitating factor is bronchial stagnation induced by the ligature, as a result of which the seemingly quite harmless organism can cause severe disease.

TABLE 1

Group	No. of rats examined	Ages (months)	No. of rats with lesions (percentages in parentheses)	No. of rats with L3 (percentages in parentheses)
1	14	less than 1 month	0	1
2	84	1–4	8 (10)	29 (36)
3	71	4–8	22 (31)	46 (65)
4	33	8–12	28 (85)	24 (73)
5	26	12–24	26 (100)	20 (77)

TABLE 2
ISOLATION OF THE L3 ORGANISM FROM THE LUNGS AFTER BRONCHIAL LIGATION AND FROM NORMAL, UNOPERATED LUNGS

Rat No.	Bronchus ligated			No operation	
	Days after ligation	Growth of L3	Extent of bronchiectasis	Rat No.	Growth of L3
1	2 (found dead)	+	0	1	0
2	2	+	0	2	0
3	4	++++	+	3	+
4	5	++++	+	—	—
5	5	++++	+	—	—
6	12	++++	++	—	—
7	12	++++	++	—	—

Another rat PPLO widely studied by myself and other workers causes an arthritis in its virulent state. The joints become edematous and contain gelatinous masses that become purulent. This organism also may be latent, or may cause an occasional swollen gland or serious lesions. Various workers have studied it in infection and have found that as a rule disease develops only when the organism is injected with adjuvants such as animal tissues and agar emulsion. Occasionally the organism appears in cancerous growths transplanted from rat to rat by passage: for example, Woglom's pyogenic virus (Klieneberger, 1939 and 1940). Several years ago I obtained two new cultures designated "Baxter" and "Jasmin." Baxter had produced a spontaneous epidemic of arthritis in the breeding stock of a London teaching hospital, and Jasmin came from rat cancer tissue sent to me from Montreal, Canada, by Jasmin. Both strains were culturally and serologically almost identical, and I used them to produce experimental abscesses in rats, a disease that could be propagated by subcutaneous injection of material from one abscess into other rats. The first abscess of a series was produced by injecting the organisms in agar emulsion. A small palpable lump developed on the third day, increased in size during the subsequent weeks, and then regressed slowly. The organism could be cultivated from the lump as long as pus was present. The rats did not seem to be ill although they were, perhaps, a little sluggish during the first weeks. At necropsy, their spleens were found to be enlarged at this stage. Rats in which regression of the local lesion had occurred had spleens of normal size at necropsy. The organisms appeared in the blood stream during the first weeks. Quite frequently the spleen, the kidneys, and the inguinal gland on the same side as the injection yielded PPLO during the first weeks after the injection. The organisms grown were identical with those injected, and no PPLO were ever obtained from these locations in control rats.

Complement fixation (CF) test of the sera from about 140 rats killed at various times before and after the injection showed that antibodies against PPLO were not present in 1/10 dilution in the sera of young normal rats. The average serum titers of the rats of 1 experimental series are plotted in FIGURE 1. The titers rise steeply to 1:2500 during the first weeks and gradually decrease during the regression period; they are specific for this rat organism.

I have also tried to produce abscesses in rats by using human genital strains. Although a small lump developed and the human organisms remained alive in it for a fortnight, no appreciable antibody to the human strains was produced. It seems that only strains pathogenic for rats can induce remarkable amounts of specific antibodies in these animals.

The rat experiments throw some light on findings in human beings. The first isolation of PPLO from human genitalia was reported by Dienes and Edsall in 1937. Since then many workers have isolated PPLO, and the frequent presence of PPLO in the discharge from patients with nongonococcal urethritis has aroused some interest and a great deal of controversy.

I consider a paper by Stokes (1955) to be of the greatest importance, for it records cultural and serologic studies carried out simultaneously in 4 patients during the course of their disease and convalescence. Stokes reports isolating PPLO from the blood of a woman with puerperal fever, from the wound and pleural fluid of a patient suffering from postoperative empyema, from high vaginal swabs in a second patient with puerperal fever, and from women with hydrosalpinx and pyrexia. In three of the patients, specific antibody, demonstrated by the CF test, appeared during the infection, reached a maximum titer at about 3 weeks during convalescence, and thereafter fell. Strains from all of Stokes' patients were serologically examined by Edward and were identified as Type 1 human genital strains, the group to which belong all but 1 of Nicol's and Edward's strains from human genitals.

My cultural and serologic work (Klieneberger-Nobel, 1959) and that of D. H. Card (1959) were undertaken with the aim of collecting further evidence on the significance of PPLO in pathological genital conditions and, particularly, in nongonococcal urethritis (NGU) in males. It should be emphasized that NGU is not defined precisely, the term being applied to all cases of urethritis where gonococci are absent. However, NGU may have various causes, noninfectious and infectious, of which PPLO infection may be only one. The demonstration of PPLO in a substantially higher proportion of NGU patients than in healthy males, however, would suggest that they may be the cause of the disease.

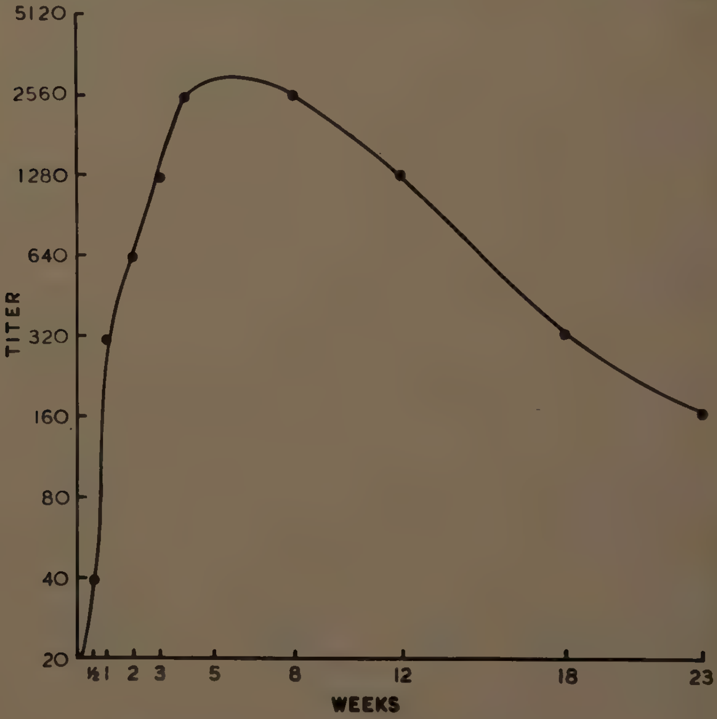

FIGURE 1. Serum titers of "Jasmin"-infected rats.

Cultural Investigation

For the cultivation of PPLO from human genitals my colleagues and I used a very rich medium containing yeast extract, desoxyribonucleic acid from thymus, a filtrate of a broth culture of staphylococci, and pooled human serum from blood donors (Klieneberger-Nobel, 1956). To inhibit bacterial growth, penicillin and thallium acetate were added according to the recommendation of Edward (1954). We found it advisable to use human serum (or ascitic fluid) for isolation of human strains, because a number of organisms did not grow on horse serum at first isolation. Specimens from the anterior urethra in males (after thorough cleansing) and from the vagina, cervix, and urethra in females were collected with a platinum loop and spread on solid media without delay. In healthy males urine was used as a specimen, since scraping the urethra did not provide satisfactory material when no discharge was present. In a preliminary investigation it was established that human genital PPLO remained viable in urine for at least 6 hours and that PPLO were isolated with the same frequency from the urine as from the discharge of patients with NGU.

Strains isolated. PPLO strains were isolated from 98 patients. All of those so examined grew equally well aerobically and anaerobically. On the preferred medium they grew as minute colonies about 0.2 mm. in diameter within 48 hours at 37° C., provided the growth was not very dense. After 3 to 5 days, discrete colonies were about 0.5 mm. in diameter and, after 7 days' incubation, were readily visible to the naked eye, often brownish yellow in color. On the isolation medium the colonies have a slightly raised dark center, under which the growth has penetrated into the agar, and a flat thin peripheral zone often marked with fine radiating lines. The edge is usually coarsely and irregularly broken, but sometimes remains unbroken. A few strains produce colonies with a fine structure consisting of translucent areas separated by darker lines, giving the appearance of openwork lace and resembling colonies of the L form of bacteria. On the simpler stock medium all these strains produce colonies with an unbroken edge.

In shaken broth cultures at 37° C., the growth as a rule is slightly and uniformly turbid, but with a few strains it is finely granular. There is no growth at room temperature (about 20° C.).

There is no danger of confusing the PPLO with bacterial L-form colonies with which they have certain properties in common, for in the few instances when colonies of PPLO resembled L colonies they were distinguishable by their greater delicacy of structure. When coarser colonies (resembling those of the stable L form of bacteria) were present, they all proved to be the so-called transformation forms induced by penicillin, and they reverted to bacterial forms when cultivated on media not containing penicillin. No true (stable) L forms were seen in any of the primary cultures of the material examined.

The number of colonies grown from the discharge or urine sediments varied. Sometimes only a few were found; in most cases a considerable number, many hundreds to many millions, developed. A case was never diagnosed as PPLO-positive unless subculture on solid medium without penicillin and thallium acetate was successful, and the strain grew characteristically in liquid media as well.

Although workers sometimes feel able to distinguish between different species of PPLO by colony type and morphology, most conclude sooner or later that no dependable distinction can be made, because these differential characters vary with minute changes in the consistency of the medium and the density of growth. Strains may vary in colony type and exhibit differences of morphology in different sectors of the same plate. Some workers have used fermentation reactions, hemolysis, and growth inhibition for differentiation of types. In our investigations none of these methods has proved to be consistently reliable.

The most constant characteristic elicited from the strains was antigenic structure. Fifty-six of our 98 strains were tested serologically by a CF method (Card, 1959). All cross-reacted to high titer with antiserum from rabbits immunized with human genital PPLO and were serologically distinct from human mouth PPLO and also from animal and saprophytic strains. We concluded that they formed a single serologic group. It is noteworthy that all but one of Nicol's and Edward's 91 human genital strains fell into the same group as did the majority of Huijsmans-Evers and Ruys' (1956) human genital PPLO strains.

TABLE 3
INCIDENCE OF PPLO IN MALE CONTROL GROUPS

	No. tested	PPLO-positive
Adults	100	3
Boys under 13 years	47	none

TABLE 4
INCIDENCE OF PPLO IN MALE VENEREAL PATIENTS

	No. of cases	PPLO-positive	Percentage positive
Total	201	51	25.5
Acute and subacute NGU	65	31	48
Untreated gonorrhea	49	15	30
Miscellaneous conditions	43	4	9.3
Reiter's syndrome	20	2	10
Chronic NGU	17	none	
Cured NGU	7	none	

Results in men. It was necessary to find a group of persons free from genital infection as controls. We assumed that boys under the age of puberty and men with a high standard of hygiene and free from symptoms would provide such controls. Of 47 urines (TABLE 3) from boys under the age of 13, none yielded PPLO. From 100 urines collected from members of the staff of the hospitals, 3 yielded PPLO. One of the men was shown to have NGU the next day; another had no symptoms, but reported previous attacks of prostatitis; no inquiry was made into the third case. We conclude that PPLO are rare in the urethras of healthy men, and that boys under the age of puberty are free of them.

From TABLE 4 it may be seen that very different results were obtained in our study of male patients from the 2 VD clinics. PPLO were isolated from 57 (25.5 per cent) of a total of 200 patients. When these patients were classified on clinical grounds, it was found that 31 (48 per cent) of 65 patients with acute and subacute NGU harbored PPLO. Of 49 patients with untreated acute gonorrhea, 15 (30 per cent) harbored PPLO. Among the other patients, suffering from Reiter's disease or miscellaneous conditions of the genitals, 10 per cent yielded PPLO. The highest proportion of PPLO isolated was found therefore in the more or less acute NGU group.

Results in women. As reported by various authors, PPLO occur more frequently among female VD clinic patients than among males. Cultural examination for PPLO was positive in 33 (56 per cent) of a total of 59 women (TABLE 5). In untreated and treated gonorrhea and in cervicitis, vaginitis, or urethritis the percentages of PPLO isolations were very high (between 80 and 90 per cent). PPLO also occurred in 2 of 6 cases of *Trichomonas* vaginitis. However, in a number of symptomless women (14) and those few examined who were suffering only from warts or genital ulcers (3) PPLO were not found. Approximately 20 per cent of obstetric (51) and gynecological patients (58) attending the London Hospital (Whitechapel district) carried PPLO, but several had *Trichomonas* vaginitis or thrush.

TABLE 5

INCIDENCE OF PPLO IN FEMALE VENEREAL PATIENTS

	No. of cases	PPLO-positive	Percentage positive
Total	59	33	56
Gonorrhea untreated	15	13	87
Gonorrhea treated	9	8	90
Cervicitis and/or vaginitis and/or urethritis	11	9	82
Trichomonas vaginitis	6	2	33
Salpingitis	1	1	
Warts, ulcers	3	none	
No symptoms at examination	14	none	

Consorts. In four pairs of consorts examined, both carried PPLO; in three pairs the organisms were not found in either partner.

In men, accordingly, PPLO are associated with acute NGU and, in women, with cervicitis, vaginitis, and urethritis. They are also frequently associated with gonococcal infection and trichomonal infestation. They are extremely rare in healthy men and boys under the age of puberty, and their transfer by sexual intercourse is likely.

Serologic Tests

We have seen from the rat experiments that pathogenic PPLO produce an antibody whose titer rises steeply at first and decreases very gradually. The titers remained between 1:2000 and 1:4000 for some time, even after the infection had cleared. As mentioned before, antibodies to the human type of PPLO have been demonstrated in infected humans. If the presence of PPLO in the human urogenital tract leads to antibody production, the incidence of antibodies in any group of people should be related to the incidence of infection. The groups of subjects in whom the incidences of VD may be expected to vary are listed in TABLE 6. As controls free of venereal disease, blood donors and children were examined. A high proportion of subjects with present or past venereal infections was to be expected in VD clinic patients. As intermediate groups, medical outpatients and people attending antenatal and gynecological departments were examined. The methods used for the CF test and the preparations of antigens are described by Card (1959). The results of the serologic survey are summarized in TABLE 6. A total of 700 patients from two VD clinics was examined. Of these, 34 per cent had PPLO antibodies in their serum. Women were positive about twice as often as men. The very high percentage of positive sera in the clinic patients corresponds closely to the percentage positive in cultural tests. The serologic data, furthermore, tally with our cultural findings as regards the frequency of PPLO infections in men and women. In contrast to these results, it was found that among the 2 control groups of children and blood donors, sera containing PPLO antibodies were rarely found. The percentage of subjects with positive titers was also low among medical outpatients and women attending an antenatal clinic in a country district where the incidence of VD is expected to be low. However, it is slightly higher than that of the blood donors and children.

TABLE 6

DISTRIBUTION OF PPLO ANTIBODIES IN HUMAN SERA

Origin or category of group examined	Total		Males		Females	
	No. tested	Positive %	No. tested	Positive %	No. tested	Positive %
VD clinic patients	700	34.2	284	18.7	416	44.5
St. Mary's Paddington	346	37.3	116	19.0	230	46.5
Whitechapel Clinic (London Hospital)	354	31.6	168	18.5	186	41.9
Medical outpatients (London Hospital)	198	6.1	111	4.5	87	8.1
Antenatal patients (Billericay)					96	6.3
Gynecological patients (London Hospital)					44	20.4
Blood donors (N.W. London Transfusion Center)	297	2.1	188	1.1	109	3.7
Children less than 13	104	2	72	2.8	32	—

The serologic findings show that positive titers against PPLO are frequent in those groups of subjects in which venereal infections abound and in which PPLO have also been cultured most commonly.

There is still a gap in these investigations, however, that must be closed by future studies. Our cultural and serologic findings are not from the same persons. It would be desirable to follow the course of NGU in men by cultural and serologic methods from the first sign of the disease to the cure, as has been done in the animal experiments. In rats, antibody titers occur only when the animals are infected with pathogenic organisms. It is assumed, therefore, that specific antibody against PPLO in humans also reveals present or past PPLO infection.

The results of our investigations are in good agreement with those of a number of other workers. Harkness and Henderson-Begg reported in 1948 that 21 (38 per cent) of 57 subacute, abacterial urethritis cases and 5 (50 per cent) of 10 cases of acute urethritis were PPLO-positive. Negative results were obtained from 65 (50 male, 15 female) "normal people." Their conclusions are: "Thus the high percentage of positive cultures in cases of NGU, their absence from normal cases, their disappearance with successful treatment, and the recurrence of positive cultures with the recurrence of lesions all tend to support the conclusions that organisms of this group are responsible for certain types of abacterial urethritis of venereal origin."

Huijsmans-Evers and Ruys (1956) also obtained PPLO 9 times from 15 acute NGU cases.

The results achieved by Nicol and Edward (1953) do not disagree with ours, although they are interpreted in a different way. They had 26 per cent positive in men with NGU and 11 per cent positive in 90 police candidates whom they regarded as "normal." This is a very significant difference, particularly if it is realized that the cases included men with acute, subacute, and chronic conditions. In our experience, most of the positives are found among the acute cases and the majority of chronic cases are negative.

The studies of Melén and Odeblad (1951) and of Melén and Gotthardson (1955) are of interest. These investigators found that 76 per cent of women with inflammatory pelvic disease and 19 per cent of women without such a condition were positive. They also investigated human sera with CF tests. High titers were obtained in 5 women who had nongonococcal salpingo-oophoritis. In 2 of them a significant rise in titer was demonstrated during the disease, and in 2 a decrease in titer occurred after recovery. In 2 cases PPLO were isolated in pure culture from ovarian abscesses.

The paper by Warthin (1948) is of great interest. Warthin reported on 4 cases of Reiter's disease that promptly reacted to streptomycin. One of 4 men had developed nongonococcal urethritis 2 weeks after exposure. A week later the patient had inflamed eyes and a swollen knee joint. The urine and the synovial fluid yielded PPLO. The condition was not influenced by sulfa drugs or penicillin, but was cured by streptomycin. Although we were never able to culture PPLO from synovial fluids, and had no appreciable number of positive serum titers in our cases of Reiter's disease, we are inclined to believe that a certain type of Reiter's syndrome, perhaps more frequent in the United States than in England, may be caused by PPLO.

Very revealing information can be gained from the excellent paper by Berg *et al.* (1957), on "acute hemorrhagic cystitis." The authors draw the clinical picture from patients in whom the diagnosis was substantiated by cystoscopy and from whom PPLO were isolated. As they point out, a bacterial pyuria has long been considered infectious (Harkness, 1950), and it is very likely that PPLO are the etiological cause. The same investigators also examined a number of sailors with and without genitourinary complaints. The percentages of positives were 65 in the first and 36 in the second group, "a highly significant difference"; for, as they point out, "there was considerable evidence, that the control groups did not actually represent a population with negative genitourinary histories or findings." Berg *et al.* also report that they found a very low percentage of PPLO positives in groups of people with "restrained venereal contacts." When, therefore, results of examinations are to be interpreted, it must be remembered that "a sexually circumspect population cannot be compared with a sexually promiscuous population."

A recent very interesting paper of Nelson (1954), throws some light on the findings in humans. Acute oophoritis and salpingitis were commonly observed in weanling mice injected I.P. with murine strains of the catarrhal type. These strains were recovered regularly from the ovary or uterus during the third to the fifth weeks after injection, but not after the twelfth week; from the vagina in the fifth week; and from the heart's blood in the first week. Nelson thinks it probable that the PPLO are carried to the ovary in the blood stream. We had evidence in our S.C.-injected rats that dissemination of PPLO into the blood occurs quite regularly during the first fortnight. A similar dissemination by way of the blood may occur in humans, although only a few reports on cultivation from the blood are found in the literature.

Among the reports on the occurrence of PPLO in the human genitourinary tract that clash with those reported thus far are those of Freundt (1956), who found PPLO more frequently in healthy men than in diseased ones. He has taken his specimens from the urethral orifice and the prepuce, however, whereas we have inserted the loop 1 to 2 in. into the urethra and taken the precaution to clean the meatus thoroughly with alcohol before this procedure. Results of examinations carried out by means of such different methods cannot be compared.

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MORPHOLOGY AND PATHOGENICITY OF L FORMS OF *CLOSTRIDIUM TETANI* INDUCED BY GLYCINE

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The L forms of *Clostridium tetani* were reported first by Dienes (1950), who obtained them with the help of penicillin. Dienes described these forms as similar in appearance and morphology to those isolated from a Gram-positive spore-bearing bacillus and from *Bacteroides*.[1,2] The reversion of L colonies to bacterial forms has not been reported for *C. tetani*. No tetanus was observed to develop in several mice injected with the L-type cultures.[2] The present study is an investigation of L forms of *C. tetani* induced by glycine, which we have found to differ in several aspects from those induced by penicillin.[3]

Materials and Methods

The two strains of *C. tetani* used in this study were obtained from the IBYS Institute, Madrid, Spain, and from the Institut Pasteur, Paris, France.

The medium employed was Prevot's VF with 1 per cent glucose and 7.5 per cent agar plus 2 to 3 cc. of 10 per cent glycine sterile solution in Weinberg tubes.[4,6] The stock cultures of the *C. tetani* strains were maintained in Prevot's medium without glycine in closed tubes.

After incubation, tubes were examined macroscopically for the presence of different types of colonies, which were observed for their cellular morphology by phase-contrast and normal microscopy. Preparations were made of washed suspensions and shadow-casted with gold-palladium for observation in the electron microscope.*

Serial transfers of such colonies to Prevot's medium with or without glycine were made and observed for reversion to bacterial colonies.

Pathogenicity tests were performed, using mice and guinea pigs. The L forms of the B colonies were injected into mice and guinea pigs after they had been washed with saline solution, centrifuged, and diluted in saline. Control animals were injected in the same way with the stock strains washed and diluted as were the L forms.

Results

Two types of colonies, designated A and B, appeared in the two strains of *C. tetani* in the Prevot's medium with glycine after 24 to 48 hours of incubation at 37° C.

The A colonies are macroscopically similar to the normal bacillary colonies (rounded and fluffy and measuring from 1 to 3 mm. in diameter). Microscopically these colonies are composed of large bodies and intermediate L forms typical of L colonies from Gram-negative bacteria.[4,5] After several days the intermediate and large bodies disappeared, being replaced by small granular forms (FIGURES 1, 2*a* to *f*, and 3*a* to *f*).[6]

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* The electron microscope used, a product of the Radio Corporation of America, New York, N.Y., was obtained from the Instituto de Optica de Madrid.

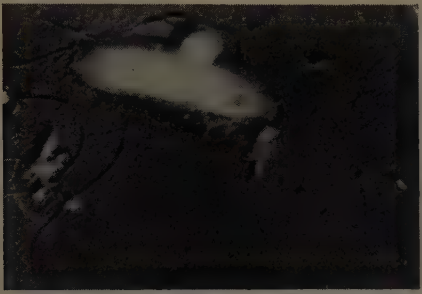

FIGURE 1. Beginning of the L cycle induced by glycine in *Agrobacterium tumefaciens.*

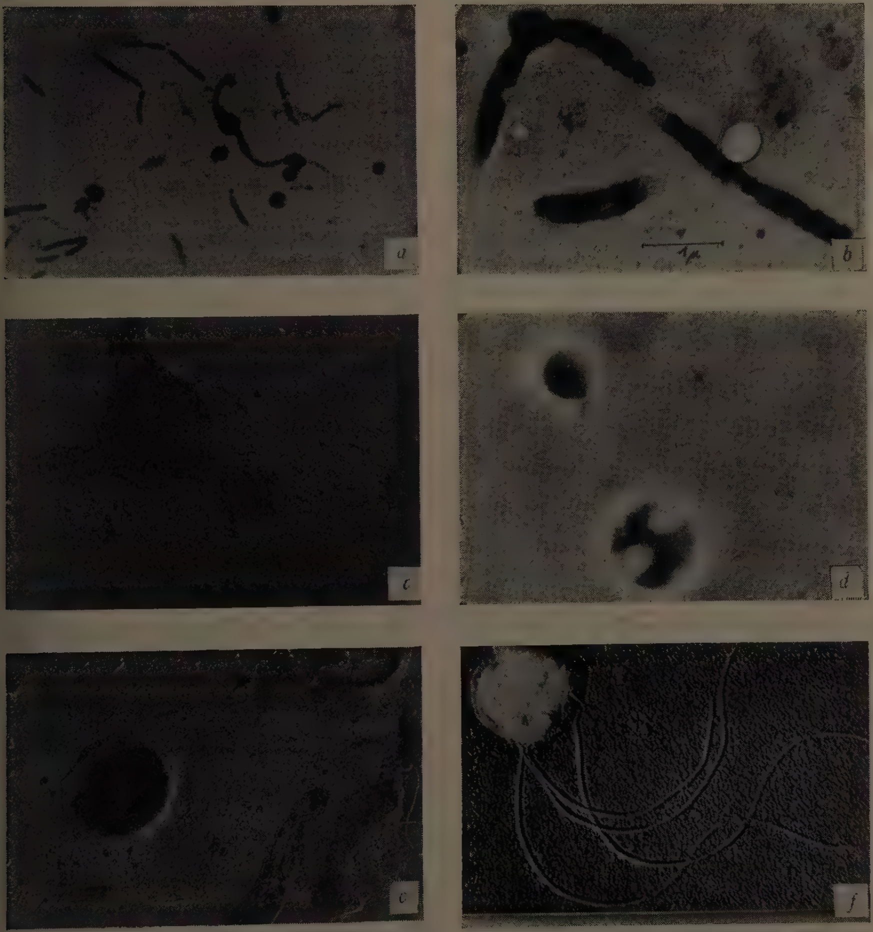

FIGURE 2. Different bacterial species treated with glycine, showing different stages of the L cycle.

One to three successive transfers from these colonies to Prevot's medium without glycine showed reversion to the typical bacillary form.

The second type of colonies (B) are more compact and lenticular, but sometimes also become fluffy. They are smaller than the A and normal colonies. Microscopically, they are composed entirely of refringent, round bodies approximately 1 to 2.5 μ in diameter, strongly Gram-positive, and arranged in clusters. When the colony became old there appeared, in addition to the round refringent bodies, larger bodies having a polygonal shape and embedded in a gelatinous matrix.

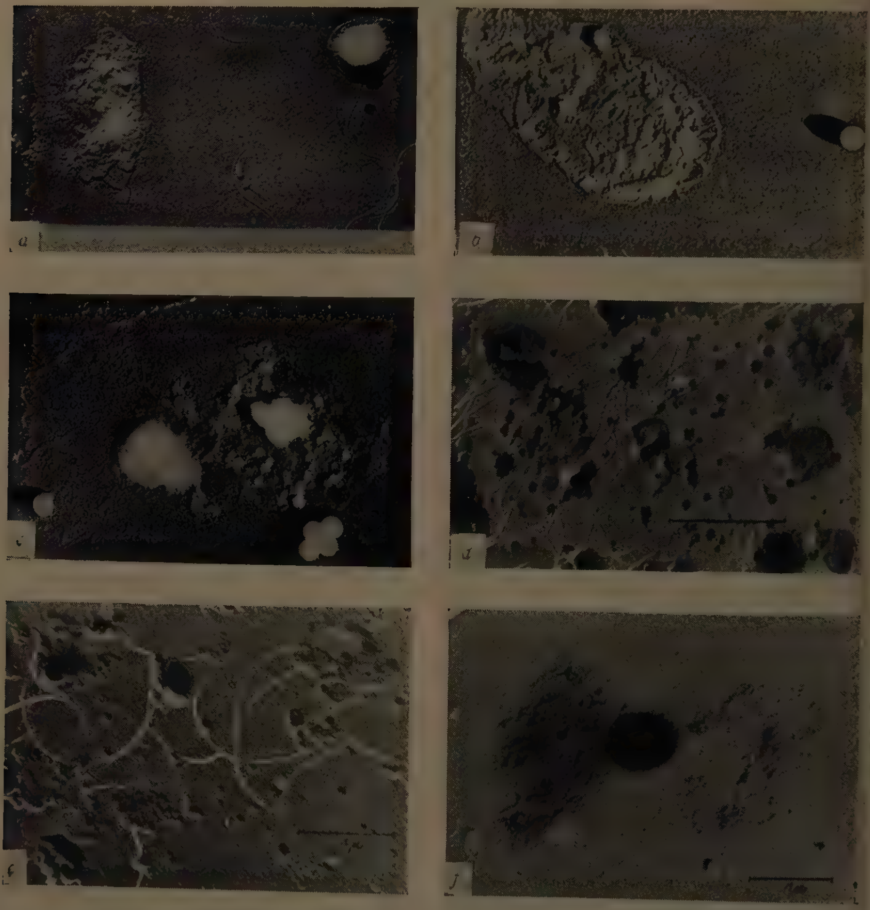

FIGURE 3. Different bacterial species treated with glycine, showing different stages of the L cycle.

Successive transfers from 1- to 4-day intervals for over one month to Prevot's medium without glycine showed no morphologic transformations; only after the twelfth to sixteenth passages were long thick filaments observed emerging from the round bodies. These filaments were pluricellular or "tabicated" as shown by special cell-wall stains, later producing the bacillary forms with all the characteristics of the normal *C. tetani* (FIGURES 4*a* to *f*, 5*a* to *f*, 6*a* and *b*).

Several guinea pigs and mice were inoculated with dilutions of the supernatant from a centrifugate of a 24-hour culture of a B colony cultivated in VF medium with glucose. Eighteen hours after the inoculation the guinea pig displayed the complete symptomatology of tetanus, while the mice died. The sediment of the same centrifugate was washed with saline and suspended in 10 ml. of saline. Six mice were inoculated subcutaneously with one tenth ml. of the suspension. All of them died in 24 hours. From 3 of the inoculated mice the L forms (the larger polygonal forms) were isolated in Prevot's medium inoculated with a small piece of subcutaneous conjunctive tissue from the site of the inoculation. In successive transfers those forms disappeared, leaving

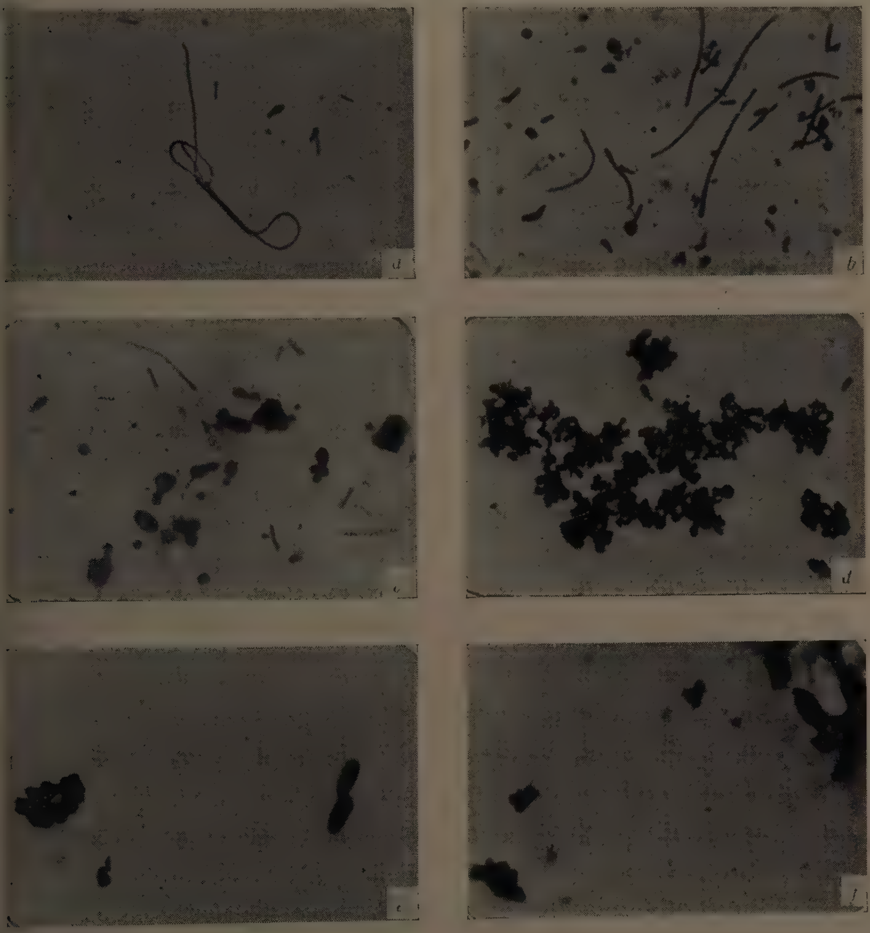

FIGURE 4. *C. tetani* in the L cycle (light microscope): *a* to *c* are from A colonies; *d* to *f* from B colonies.

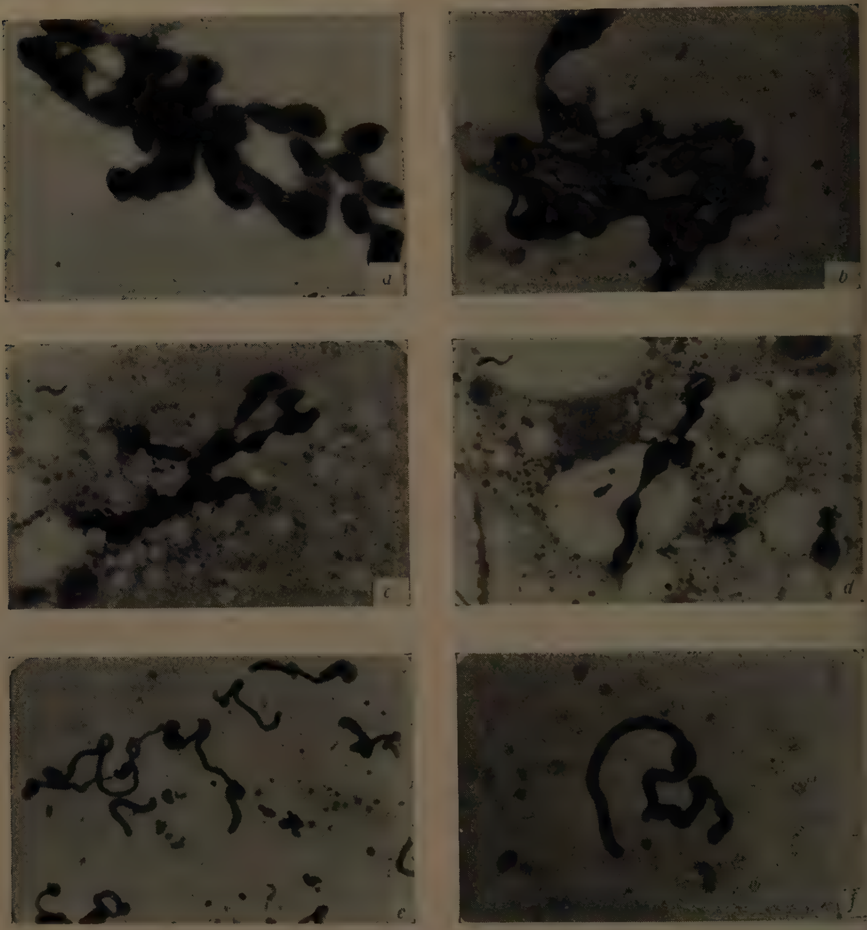

FIGURE 5. *C. tetani* in the L cycle (light microscope): *a* and *b* are from B colonies; *c* and *d* are from smears of inoculated mice; *e* and *f* represent the evolution from the L form through filaments to the normal form.

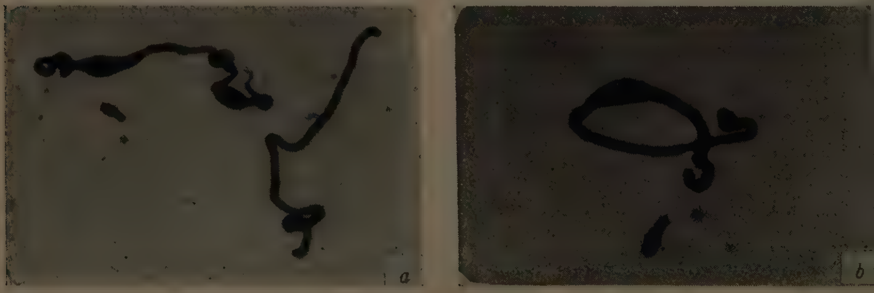

FIGURE 6. *C. tetani* in the L cycle (light microscope). Both *a* and *b* represent the evolution from the L form through filaments to the normal form.

nly the rounded refrigent forms in clusters. The normal bacillary form never vas recovered from any of these cultures of subcutaneous tissue.

These experiments were repeated several times, using chiefly the supernatant r fraction with the toxin only and, in every case, with controls.

We observed the L forms in smears made with subcutaneous tissue from the noculated mice.

In our work with L forms from various types of bacteria[4,5,6] we never observed colonies formed by such elements as those of the B type in *C. tetani* but, s these appear only at the same time as do the normal L colonies by the influence of glycine, it seems that they belong to the L cycle. Furthermore, we ave found the same type of colony in other anaerobic bacteria in which we are ow studying the L cycle.

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THE OCCURRENCE OF ORGANISMS OF THE PLEUROPNEUMONIA GROUP IN THE EXTERNAL GENITALIA OF FEMALE CHILDREN

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It has been demonstrated that pleuropneumonialike organisms (PPLO) can be found in the genital tract of healthy adult women; apparently they form part of the normal bacterial flora of the vagina. The literature on this subject has been reviewed by Sabin (1952). Several authors (Dienes and Smith, 1942; Klieneberger-Nobel, 1945; Salaman *et al.*, 1946; and Randall *et al.*, 1950) have observed a greater incidence of PPLO in the genital tract of adult women with inflammatory processes than in the genital tract of normal females; nevertheless, no definite etiological relationship has been established between the organisms isolated and these pathological conditions.

It is the purpose of this paper to report the incidence of organisms of the pleuropneumonia group in the external genitalia of a limited number of female children, only some of whom had nongonococcal vaginitis.

Clinical Material

One hundred and seven female children admitted to the Hospital Infantil de Mexico were studied. Eighty-seven of the cases were suffering from various diseases other than vaginitis, and their genital organs were found normal at the clinical examination. The other 20 patients had nongonococcal vaginitis as the chief complaint. All the patients in the vaginitis group had previously been given penicillin and other antibiotics for varying periods. The ages of the children are shown in TABLES 1 and 2.

Method

Specimens of the discharge present in the external genitalia of the children were collected, using sterile cotton swabs. Each specimen was inoculated by rubbing the swab into 10 ml. of PPLO Difco fluid medium containing 1 pe cent Bacto-PPLO serum fraction (Difco 0441-63), 0.001 per cent crystal violet 0.0025 per cent potassium tellurite, and 0.1 per cent sodium sulfadiazine. The crystal violet and potassium tellurite were added on the recommendation o Smith *et al.* (1950). Sodium sulfadiazine was added to the medium in orde to reduce further the large number of enteric bacteria found in the specimen at the beginning of this study. Sulfonamides in the concentration used hav been reported to have no effect on the growth of PPLO (Salaman *et al.*, 1946)

After 3 to 5 days of incubation at 37° C. the tubes containing the fluid mediu were shaken and 1 drop of the medium was transferred to plates of PPLO Difc serum fraction-enriched medium with 1.5 per cent agar. Crystal violet, tellu rite, and sulfadiazine were omitted for this phase of the work. The plate were sealed and incubated in an inverted position for 2 to 5 days at 37° C. an then examined under the low power of the microscope. When minute colonie measuring approximately 0.1 to 0.5 mm. in diameter were observed, a piece o agar containing the colonies was inoculated into PPLO Difco serum fraction-enriched fluid medium without inhibitors. If, after 2 to 5 days of incubation, no bacterial growth was observed, smears were prepared from centrifuged sediments and stained by the Giemsa method. Those cultures showing some of the polymorphic structures described as characteristic of the pleuropneumonia group (Sabin, 1952) were considered positives.

TABLE 1

INCIDENCE OF RECOVERY OF ORGANISMS BELONGING TO THE PLEUROPNEUMONIA GROUP AND TO THE FAMILY ENTEROBACTERIACEAE FROM THE EXTERNAL GENITALIA OF 87 FEMALE CHILDREN WITH NORMAL VAGINAS

Age (years)	No. of cases studied	Incidence of organisms growing at primary isolation on PPLO fluid medium* (per cent)	
		Pleuropneumonia group	Family Enterobacteriaceae
0–2	12	0	100
3–4	12	0	83
5–6	16	6	44
7–8	8	12	12
9–10	23	26	17
11–12	16	19	6
All ages	87	12.6	40.0

* The medium contained crystal violet (0.001 per cent), potassium tellurite (0.0025 per cent), and sodium sulfadiazine (0.1 per cent).

TABLE 2

INCIDENCE OF ORGANISMS OF THE PLEUROPNEUMONIA GROUP IN THE EXTERNAL GENITALIA OF PATIENTS WITH NONGONOCOCCAL VAGINITIS AND FEMALE CHILDREN WITH OTHER CLINICAL SYNDROMES*

Nongonococcal vaginitis present	No. of cases studied	Incidence of pleuropneumonia organisms	
		No. of positive cases	Percentages
Yes	20	6	30.0
No	59	8	13.5

* All cases were in the 3- to 11-year-old age group.

When gross bacterial growth was obtained in the plates, the nature of the microorganisms was determined using standard techniques (Dubos, 1952).

Results and Discussion

In spite of the inhibitors contained in the medium, microorganisms belonging to the family Enterobacteriaceae, particularly *Proteus*, often grew so profusely on the plates that they prevented the observation of PPLO colonies. These will be the only contaminants considered in the results. Gram-positive cocci, even if observed on occasion, were not as troublesome.

TABLE 1 shows that, using the described techniques, 12.6 per cent of 87 control female children up to 12 years of age carried organisms of the pleuropneumonia group in the external genitalia. These organisms were not detected in the external genitalia of any of the children less than 4 years of age. It is interesting to point out that microorganisms of the family Enterobacteriaceae were isolated from most of the specimens taken from these cases. In older children, 5 to 12 years old, there was a decrease in the incidence of enteric bacteria in vaginal samples, and it is interesting to note a concurrent increase of PPLO (6 to 26 per cent of 63 cases) in this age group. These findings suggest that the absence of PPLO in the cultures taken from the youngest children may have been due to the overgrowth of microorganisms of enteric origin, common in the external genitalia at this age. The difficulty of demonstrating PPLO in the presence of abundant bacterial growth has been shown by Dienes (1947). This study further emphasizes the need for a more effective medium.

The results of this study show that PPLO are present in the vaginal tracts of young children, a finding that, to our knowledge, has not been reported previously. However, the data presented in TABLE 2 indicate that in this limited survey no significant difference existed in the incidence of PPLO isolated from individuals with vaginitis or from patients with other syndromes. Since the number of cases is small, it is difficult to draw any concrete conclusions.

Acknowledgment

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THE ORIGIN OF PPLO FOUND IN RECTAL CULTURES

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The pathogenicity of pleuropneumonialike organisms (PPLO) for humans is most evident when they produce, in ordinarily sterile organs or tissues, inflammatory lesions such as acute hemorrhagic cystitis[1] and isolated abscesses.[5] The presence of these organisms in body orfices that normally contain a mixed flora is more difficult to interpret. They are very common in the mouth and lower genitourinary tract. Some preliminary observations reported here indicate that they are found less commonly in anal cultures under circumstances suggesting an origin in the genitourinary tract.

Methods

Cotton swabs were autoclaved with a small amount of nutrient broth in cotton-plugged glass tubes. At the time cultures were taken, the broth was squeezed out of the swab against a gloved finger to serve as lubricant. The swab, held against the forefinger, was inserted through the length of the anal sphincter. It was withdrawn, partly rotated, and reinserted. Withdrawn again, it was immediately stroked with rotation on both halves of an ascitic agar plate. To one half, a small amount of penicillin had been added. A bacterial loop was used at once to stroke out the culture, and the paraffin-sealed plate was put into an incubator within one hour (usually sooner) after having a culture of *Bacillus prodigiosus* implanted on the inverted top to produce an anaerobic atmosphere.

Results

Case studies. Two cases of urethral gonorrhea associated with PPLO in rectal cultures of venereal contacts led to these studies.

Patient 1, a 29-year-old, single businessman, was first seen in October 1956 because of a proctitis. Cultures were taken and revealed abundant coliform and nonhemolytic streptococci and abundant PPLO. A repeat culture 2 months later was negative for PPLO. He was again seen in May 1958 with slight rectal discomfort and itching. An anal swab yielded PPLO on culture.

Patient 2, a 24-year-old single student, was first seen in May 1958 with a purulent urethral discharge that appeared 6 days after an initial rectal contact with Patient 1. The culture grew out *Neisseria gonorrhea*, but no PPLO.

Patient 2 appears to have contacted urethral gonorrhea from Patient 1, who showed PPLO, but not *N. gonorrhea* on anal culture.

Patient 3, a 28-year-old businessman, was seen because of a minimal watery discharge of 11 days' duration, which appeared five days after rectal exposure. On culture, abundant *N. gonorrhea* were found, but no PPLO. This patient was treated with penicillin.

Patient 4, a 24-year-old clerk, was seen because he had been exposed rectally to the urethral gonorrhea of Patient 3 (he denied other contacts) a few days before treatment was given. He had no symptoms, and there were no abnormal physical findings. A culture of an anal swab was negative for *N. gonorrhea*, but produced abundant PPLO.

TABLE 1
PPLO IN MOUTH AND RESPIRATORY TRACT

Investigators	Year	Patients			Source
		Sex	Number (infected/ total)	Percentage infected	
Beveridge *et al.*[3]	1946	M & F	0/57	0	Antral washings
		M & F	0/70	0	Excised tonsils
Smith & Morton[28]	1951	M & F	46/100	46	Saliva
Morton *et al.*[16]	1952	M & F	20/26	77	Normal saliva
			3/13	23	Normal throat
			14/27	52	Lab sputa samples
			0/10	0	Bronchoscopy samples
			49/144	34	Throat swabs
Nicol & Edward[20]	1953	M	0/97	0	Mouth
		F	0/5	0	Mouth
Over-all			132/549	24	

TABLE 2
PPLO IN RECTUM AND IN STOOLS

Investigators	Year	Patients		Source
		Number (infected/ total)	Percentage infected	
Harkness[10]	1950	4/30	13	Normal subjects; anal swabs
		2/13	15	Sigmoidoscopy
Morton *et al.*[16]	1952	1/27	4	Fecal specimens
Nicol & Edward[20]	1953	6/109	6	Male
		12/18	67	Females, mostly with proctitis
Berg *et al.* (this paper)	1959	2/30	7	Males, no rectal symptoms
		0/3		Females, no rectal symptoms
		0/6		Anal fissures
Over-all		27/236	11	
		15/218	7	
Harkness[10]	1950	2/9		Passive homosexuals
Berg *et al.* (this paper)	1959	3/14		Passive homosexuals
		1/1		Condylomata acuminata
Over-all		6/24	25	

Patient 4 was exposed to acute (although atypical) urethral gonorrhea, but his anal cultures produced PPLO and not *N. gonorrhea*. Cultures from Patient 3, the contact, produced *N. gonorrhea* and not PPLO.

Patients without rectal complaints. Thirty male patients (average age, 48 years), who were patients on a general medical ward or in a general surgical clinic, had anal swab cultures taken. Many of these patients had hemorrhoids, although few complained of them. There was no other local pathology. Two patients had PPLO on culture. One of these was a 63-year-old married patient with a history of lues and of hospitalization for undifferentiated carcinoma of the lung. The other was a young man with cat-scratch fever. Neither case had rectal findings except for a small hemorrhoidal tag in the patient with carcinoma of the lung. Three female patients had negative cultures.

TABLE 3
PPLO IN FEMALE GENITOURINARY TRACT

Investigators	Year	Normals		Patients	
		Number (infected/total)	Percentage infected	Number (infected/total)	Percentage infected
Klieneberger-Nobel[12]	1945	2/45*	4	18/51	35
				5/5**	100
Beveridge *et al.*[3]	1946	17/101	17	3/11†	27
Salaman *et al.*[25]	1946	1/17	6	8/18	44
Dienes *et al.*[7]	1948			51/214	26
Harkness & Henderson-Begg[11]	1948	0/15	0	12/46	26
Randall *et al.*[22]	1950			39/76††	51
				39/224	17
Melén & Odeblad[15]	1951	29/154	19	57/66††	86
	1952	8/58	14		
Morton *et al.*[16]	1952	0/12	0	8/27	30
Freundt[9]	1953	51/156	33	116/187	62
Nicol & Edward[20]	1953	9/40	22	78/109	72
Ruiter & Wentholt[24]	1953			10/14	71
Somerson *et al.*[29]	1955			19/45‡	42
Röckl & Nasemann[22a]	1956			62/117	53
Suchanová & Šebek[30]	1956	(243)	5.5		39
Durel & Siboulet[7a]	1957	7/31	22	184/298§	62
Nermut *et al.*[19]	1958	49/134	37	230/571	40
Over-all		173/763	23	939/2079	45

* Normal pregnancy.
** Abnormal pregnancy.
† Contacts of men with nonspecific urethritis.
†† Inflammatory disorders.
‡ Males had contact with gonorrhea; no *N. gonorrhea* was found.
§ Prostitutes.

Patients with rectal complaints. Six patients (2 men, 4 women) with anal fissures did not reveal PPLO on culture. A 17-year-old male student with condylomata acuminata did have abundant PPLO on culture.

Patients with venereal contacts. Fourteen male volunteers (average age, 33 years) who had recently engaged in unusual sexual practices had anal swabs taken. Of these, 3 showed PPLO on culture. Two of these men had had rectal contact within 1 week of culture; the third denied such exposure at any time, and had not had any veneral disease. In addition, 10 of the remaining 11 men in this group who had negative cultures for PPLO had had rectal contact, although only 4 had had this contact not more than 1 month before examination.

These 14 men included patients 1 and 3 (above). Patient 1 had a negative culture for PPLO and Patient 3 had rare PPLO at this time.

A history of venereal disorders was obtained from 8 of the 14 men; this included 2 diagnoses of syphilis, 6 of gonorrhea, 5 of nongonococcal urethritis, and 2 of rectal condylomata. Furthermore, there was considerable female venereal exposure, so that the conclusion cannot be drawn that PPLO are disseminated from an exclusively male reservoir.

TABLE 4
PPLO IN MALE GENITOURINARY TRACT

Investigators	Year	Normals		Patients	
		Number (infected/total)	Percentage infected	Number (infected/total)	Percentage infected
Dienes & Smith[6]	1942			4/44	9
Beveridge[2]	1943			4/24	17
Beveridge *et al.*[3]	1946	0/67	0	14/70	20
Salaman *et al.*[25]	1946	4/28	14	8/96	8
Williams[31]	1946			10/19†	53
Dienes *et al.*[7]	1948			6/71	8
Harkness & Henderson-Begg[11]	1948	0/50	0	14/31††	45
				77/206‡	37
Harkness[10]	1950	7/68	10	50/602§	8.3
Morton *et al.*[17]	1951			9/26	35
Melén & Linnros[14]	1952	10/60	17	11/61	18
Nicol & Edward[20]	1953	14/110	13	36/140	26
Ruiter & Wentholt[24]	1953			8/32	25
Shepard[27]	1954	1/55	2		
Berg *et al.*[1]	1957	36/98	37	57/88	52
Durel & Siboulet[7a]	1957	5/28	18	85/631	14
Edward[8]	1957	5/28*	11	36/170	26
		14/116**	13		
Peoples *et al.*[21]	1957			5/34	15
Freundt[9a]	1957		49		35
Shepard[26]	1957	19/57	33	30/42	71
Over-all		115/765	15	464/2387	19
				329/1154	29

* No sexual contact.
** Urogenital anomaly.
† Inclusion bodies.
†† Acute abacterial.
‡ Subacute abacterial.
§ Other.

Discussion

The significance of the presence of PPLO in cultures from anal swabs is not entirely clear. PPLO are found in the mouth frequently (TABLE 1) and, if scrapings of the teeth are made from just under the gum margin, the incidence is much higher. The mouth cannot be the origin of rectal strains, however, for oral strains have morphologic differences distinct from rectal strains. Actually, we have not cultivated either oral or other strains from numerous stool cultures, and others have had similar results.[2,14,16] The rectal strains we have isolated are indistinguishable from genital strains. Sewage strains[14] are dissimilar.

On the basis of the morphologic characteristics, it can be concluded that rectal strains originate in the genitourinary tract. Further evidence can be deduced from our observations described above. In clinic patients and in hospitalized patients without rectal complaints the incidence of PPLO in anal swabs was 7 per cent, which is in good agreement with findings reported by other investigators (TABLE 2). In the genitourinary tract, the pooled incidence is much higher (TABLES 3 and 4). It is somewhat surprising that the incidence in anal-swab cultures is not higher, considering the proximity of the genitourinary tract and the rectum, and the expected disseminating effect of fomites. In cases where there was an increased probability of direct urethral contact, the incidence is increased to about 25 per cent, and this agrees well with observations made by Harkness of a similar population. These results suggest that proximity and promiscuity are the major factors that contribute to the finding of PPLO in rectal cultures.

TABLE 5
COINCIDENT PPLO AND *N. GONORRHEA*

Investigators	Year	Males		Females	
		Number (infected/total)	Percentage infected	Number (infected/total)	Percentage infected
Klieneberger-Nobel[12]	1945			3/9* 14/19†	33 74
Salaman *et al.*[25]	1946	12/35 2/34*	34 6		
Dienes *et al.*[7]	1948			2/11	18
Harkness & Henderson-Begg[11]	1948	12/139	9	3/18	17
Harkness[10]	1950	29/107	27	15/19	78
Randall *et al.*[22]	1950			6/6	100
Melén & Linnros[14]	1952	1/31† 3/36*	3 8		
Nicol & Edward[20]	1953	4/36	11	38/42	91
Rubin *et al.*[23]	1954			18/24	75
Somerson *et al.*[29]	1955			33/41	80
Edward[8]	1957	4/36	11		
Over-all		67/454	15	114/165	69

* Old gonorrhea, negative cultures.
† Cultures positive for gonorrhea or syphilis.

The relationship between gonorrhea and the presence of PPLO on rectal culture should not be taken as evidence for transformation of one to the other, in spite of their frequent coincidence (TABLE 5). The finding of satellite colonies of PPLO about colonies of *N. gonorrhea* and other organisms[4,6,17] is now thought to be due to the diffusion of growth factors for PPLO from the bacterial colony.

These observations do not suggest that PPLO are responsible for proctitis in a small group of cases. The presence of PPLO in pure culture from a peri-rectal abscess would be much better evidence, and Klieneberger-Nobel has had such a case.

Conclusions

(1) The incidence of PPLO in anal areas is lower than in the mouth or genitourinary tract.

(2) Rectal PPLO probably originate from the genitourinary tract.

(3) The relationship between PPLO and *N. gonorrhea* is a puzzling one, but their frequent coexistence is probably a reflection of a common mode of transmission.

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THE INCIDENCE AND CLINICAL SIGNIFICANCE OF PLEUROPNEUMONIALIKE ORGANISMS IN THE GENITAL TRACT OF THE HUMAN FEMALE

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Although the first isolation of pleuropneumonialike organisms (PPLO) from a pathological process in man was made from the female genital tract (a Bartholin's gland abscess) by Dienes and Edsall[1] in 1937, the possible pathogenicity of PPLO and their relationship to pathological conditions in the female genital tract are still unresolved.

Incidence of PPLO in the Genital Tract of the Human Female

For practical purposes, organisms resembling pleuropneumonia organisms that are isolated from various sources without any apparent relationship to a known bacterium are designated PPLO. When similar forms are isolated from a bacterial culture and bear some relationship to it, the forms are referred to as L forms, and their colonies as L-type colonies.[2] However, reports of the incidence of these organisms in the human female genital tract have not always indicated whether L forms or PPLO have been dealt with; hence, no distinction between the two will be made here.

With respect to the incidence of PPLO in the female genital tract, it is noteworthy that, if cultures from the urethra or cervix are positive for PPLO, the PPLO will appear almost invariably in the vaginal culture.[3] Therefore, cultures from the vagina alone give a good indication whether PPLO are present in the lower genital tract. Melén and Odeblad[4] have presented some evidence to suggest that cultures for PPLO are more likely to be positive in the first ten days of the menstrual cycle, but Somerson *et al.*[5] were unable to confirm this.

PPLO have been found in the healthy lower genital tracts of females of all ages. They have been reported in girls less than 3 years of age, and in women as old as 81. For example, Freundt[3] found the organisms in cultures from the vulva in 6 per cent of 50 girls less than 3 years old. None of the 50 had any evidence of genitourinary inflammation. Randall *et al.*[6] isolated PPLO from the cervices of women ranging in age from 15 to 81 years. They found them most commonly in subjects between 15 and 40, that is, in the era of active menstrual and sexual life. The over-all incidence of PPLO reported for healthy females has ranged from none to 17 per cent. Harkness and Henderson-Begg[7] were unable to demonstrate any in 15 normal women. Morton *et al.*[8] also failed to culture any PPLO from the cervices of 12 healthy women, and Salaman and his co-workers[9] found them only once (6 per cent) in 17 normal women. However, Beveridge *et al.*[10] found a 16.8 per cent incidence in 101 apparently normal women. Randall *et al.*[6] obtained 17 per cent positive cultures for PPLO in 224 women free of evidence of genital-tract infection.

Inflammatory conditions of the genital tract appear to be associated with a much higher incidence of PPLO. Melén and Odeblad[4] obtained positive cultures from 76 per cent of 78 women with such inflammatory lesions as cervicitis, vaginitis, and salpingitis. These same workers found positive cultures in only 19 per cent of 78 women with noninflammatory disease of the genital tract. Klieneberger-Nobel[11] found PPLO in only 2 (4.4 per cent) of normal pregnant women, but in 40 per cent of 45 women attending a venereal disease clinic. Ruiter and Wentholt[12] isolated PPLO from 71 per cent of 14 women with leukorrhea. Freundt[3] found PPLO in 69 per cent of 146 women with abnormal vaginal discharge, compared with 29 per cent in 175 subjects without leukorrhea. Randall *et al.*[6] have confirmed this statistically significant correlation between PPLO and inflammatory conditions in the genital tract. Gonorrhea, in particular, appears to be associated with the presence of PPLO in as many as 90 per cent of patients, according to Nicol and Edward.[13] We, too, have found this high incidence.[5,14] Similarly, *Trichomonas* vaginitis appears to be associated with a high incidence of PPLO.[9,13]

The fact that PPLO grow better in an alkaline environment may be an explanation for their scarcity in the healthy vagina. Freundt[3] found a distinct relationship between *p*H of the vagina, the vaginal flora, and the presence of PPLO. The PPLO usually were absent in healthy vaginas, which have a lower *p*H (3.7 to 5.0) and a flora of Döderlein's bacillus exclusively. A similar relationship between the presence of PPLO and lactobacilli was noted by Huijsmans-Evers and Ruys.[15]

Venereal Transmission

The question of venereal transmission of PPLO remains unanswered. Melén and Odeblad[4] failed to find the organisms in 13 virgins (mean age, 21 years), but Freundt[3] found PPLO in 4 of 6 adult virgins; however, 2 of the 4 had vaginitis and leukorrhea. Dienes and Smith[16] reported 2 instances in which there was a possibility of transmission of PPLO between husband and wife, and a third case in which a woman developed an acute vaginitis 4 days after coitus, with an abundant growth of PPLO derived from the discharge. Morton *et al.*[17] reported 2 instances in which similar types of PPLO were isolated from both husband and wife. Ruiter and Wentholt[12] isolated identical types of PPLO from a woman with severe leukorrhea and from her husband's prostatic fluid. Mankle[18] reported a case in which it appeared that the male harbored an infection with PPLO, transmitted the infection to a female sex partner, and later was reinfected by her.

Relationship to Previous Penicillin Therapy

The possible relationship of previous penicillin therapy to the presence of PPLO must be considered also, because of the ability of penicillin to produce the L forms of many bacteria. Schaub and Guilbeau[19] isolated PPLO from the *post partum* uterus of 3 per cent of untreated patients and from 20 per cent of patients who had received penicillin. Randall *et al.*[6] found PPLO in the cervices of 70 per cent of 34 women who had received penicillin, compared with 26 per cent in 300 consecutive gynecologic patients, and 17 per cent in women with noninflammatory disease of the genital tract.

The Relationship of PPLO to Pathological Conditions of the Genital Tract

The much higher incidence of PPLO in association with leukorrhea and inflammatory disease of the genital tract, including gonorrhea, has been mentioned. This suggests a relationship between PPLO and inflammatory pathology of the genital tract, but not necessarily one of cause and effect. Occasionally, PPLO have been found in abundance in inflammatory and pyogenic lesions of the genital tract. Among these were Bartholin's gland abscesses (6 cases), pelvic abscesses associated with puerperal infection or with salpingitis,[20] tubo-ovarian abscesses[6] (2 cases), and a periurethral abscess.[21] PPLO have been isolated from the blood stream and cervix of a woman during a febrile illness that had its onset 24 hours after childbirth.[22] The appearance of the organisms in the blood stream at the onset of the acute illness, and their disappearance coincident with recovery of the patient, suggest that the organisms were the cause of the illness. Stokes[23] has reported a similar case of puerperal sepsis.

It would appear that in the female genital tract PPLO usually act as commensals or opportunists.[13,16] However, they can establish themselves and act as pathogens on surfaces or in foci where the environment is suitable for their multiplication—such as a locus minoris resistentiae. Rarely, sepsis may develop if they are introduced into the blood stream from the genital tract as a result of trauma, such as that accompanying childbirth.

Summary

In the human female, if cultures from either the urethra or cervix are positive for PPLO, the organisms almost invariably will be found in vaginal cultures. Therefore, cultures from the vagina alone will give a good indication whether PPLO are present in the lower genital tract.

The incidence of PPLO in the healthy lower genital tract of women is reported to vary from 0 to 17 per cent. PPLO have been found in the genital tracts of infants and in the aged, as well, although the greatest incidence is in females between 15 and 40 years of age. It is more common in married women. Prior penicillin therapy may result in an increased incidence of PPLO in the genital tract.

In inflammatory disease of the genital tract, or with leukorrhea (specific, as from *Trichomonas vaginalis* or *Neisseria gonorrhoeae*, or nonspecific), the incidence of PPLO is often 70 to 80 per cent. The relatively low *p*H of a healthy vagina and its abundance of Döderlein's bacilli appear to be associated with an absence of PPLO. In most instances in which PPLO are found in the presence of inflammatory disease, the PPLO appear to be opportunists or commensals, rather than the responsible pathogens. However, occasional cases of bartholinitis, vaginitis, cervicitis, salpingitis, and puerperal septicemia have been associated with pure cultures of PPLO. In a few instances following sexual exposure to a male known to harbor PPLO in his genital tract, women have developed acute symptoms of genital-tract inflammation, and PPLO have been found as the predominant or sole organism in the inflammatory lesions.

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UVEITIS AND PLEUROPNEUMONIALIKE ORGANISMS

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The pleuropneumonialike organisms (PPLO) have been suggested only recently[1] as possible etiological factors in nongranulomatous types of inflammations of the iris, ciliary body, and choroid, which comprise the so-called uvea of the eye. Symptoms, clinical signs, and course of the disease usually serve to differentiate between granulomatous and nongranulomatous types of uveitis. In 1941 Guyton and Woods[2] observed that this type of inflammation can be explained best on the basis of local ocular hypersensitivity developing from a transient bacteremia that may arise from a clinically noninfected surface. A brief review follows of the reported findings relative to the suspected pathogenicity of the PPLO for human beings, especially from the ophthalmological viewpoint.

Bridré and Donatien,[3] when they described the syndrome of agalactia in sheep and goats in 1923, noted corneal lesions (keratitis) and isolated the PPLO from conjunctival sacs and joints, as well as from other areas. It appears that this represents the first recovery of these organisms from a portion of the ocular apparatus in the living animal.

Certain endogenous strains of PPLO have been shown to produce, in mice, conjunctivitis and migratory polyarthritis in addition to other inflammations.[4]

When Dienes and Edsall[5] isolated PPLO from a laboratory worker with an abcess of Bartholin's gland, it marked the first isolation from a human source. Since that time isolations from different portions of the human body have been made by many observers. Smith and Morton[6] found these organisms in the saliva of 46 per cent of 100 and in the throats of 33.6 per cent of 144 normal subjects. Shepard[7] reported positive prostatic fluid cultures in 56 per cent of 215 apparently normal males. Dienes *et al.*[8] found the organisms in the genitalia of 26 per cent of 222 normal females, while Salaman[9] found PPLO in 75 per cent of females with various inflammations of the genitourinary tract and in 6 per cent of normal women. Harkness[10] considered the organisms directly related to many types of abacterial or nonspecific urethritis in the male. Gynecologic disorders have also been ascribed to the PPLO.[11]

On the basis of positive blood cultures for PPLO, 3 cases of purpura,[12,13] a septicemia from a human bite,[14] and a subacute bacterial endocarditis[15] have been attributed to this group of organisms.

In 1942 Dienes and Smith[16] linked the PPLO to Reiter's syndrome, of which the classic triad, diagnostically, is conjunctivitis, urethritis, and arthritis, with occasional uveitis, keratitis, skin lesions, and other findings. In this regard it is of more than casual importance that Reiter's original case report[17] reveals that his patient developed uveitis (iridocyclitis), macular keratitis, and episcleritis about two months after the initial symptom complex.

Paronen,[18] in perhaps the largest single series of Reiter's syndrome (344 cases), found that 89 per cent had had conjunctivitis, 6.4 per cent uveitis (iritis), and 6.8 per cent keratitis. The iritis was of a fairly mild nongranulomatous type lasting from 3 weeks to 5 months and was a rare initial finding. When present in his series, the uveitis appeared 10 days to 17 months after the initial conjunctivitis. This latent period is compatible with the time necessary to develop a hypersensitive state.

Dienes *et al.*[8] reported 3 cases of recurring iritis and one of repeated keratitis in 9 patients with Reiter's syndrome. Hollander *et al.*[19] found 2 patients with superficial keratitis and 1 with moderately severe iritis in 11 patients with this syndrome.

Morton *et al.*[20] in 85 consecutive urology patients, found that 12 per cent had or had had iritis.

It was recognized many years ago that prostatitis and arthritis, which frequently may be abacterial by the usual culture methods, are not uncommonly associated with uveitis. Consequently, survey for existent foci of infection in the genitourinary tract, as well as the paranasal sinuses, teeth, and other areas, has become a rather well-established procedure in the diagnostic evaluation of a patient with uveitis. The apparent oculogenital affinities of certain microorganisms focuses the special attention of this survey on the genitalia.

Clinical Study

Specimens of prostatic secretions obtained by massage and of vaginal secretion obtained by aspiration were examined for bacteria by stained smears and various types of culture, in addition to PPLO culture, as explained in a previous paper[1] in which the 14 cases below were reported.

A series of patients seen consecutively with a diagnosis of uveitis were studied in this manner as part of their "focus of infection" examination. When positive cultures were obtained for PPLO, 250 mg. of oxytetracycline or tetracycline was given 4 times daily for 1 week, in addition to the usual topical eye medication.

Of 14 patients (including 1 female) thus studied, 7 had positive PPLO cultures without significant routine bacterial findings. Three of the 7 negative cultures were from patients in whom direct eye trauma was immediately related to the onset of the eye disease. In another such patient, recurrent *Micrococcus aureus* furunculosis was present and considered of etiological significance. One patient with negative culture was undergoing long-term streptomycin therapy for pulmonary tuberculosis at the onset of nongranulomatous uveitis; this may explain his negativity.

Of the 7 patients receiving the antibiotic (known to be one of the most effective agents in eradicating the PPLO[21]), repeat cultures made after the 1 week of therapy were all negative for PPLO. All but 1 of these patients had had previous uveitis attacks. Two had the granulomatous type of uveitis and did not show any perceptible reaction to antibiotic therapy. However, the remaining 5 seemed clinically to respond more promptly and required a period of topical therapy shorter than that usually encountered in similar cases treated with topical medication only.

Following isolation of PPLO from the prostatic secretion (and also from the conjunctiva) in a single patient with Reiter's syndrome, suspicion was aroused as to whether uveitis may be related to a more proximate location of the organisms. In an effort to determine the normal incidence of PPLO in conjunctival cul-de-sac fluids, thirty noninflamed right eyes of patients presenting for refraction were cultured, with not one positive culture resulting.

However, in twenty-five consecutive untreated cases of acute simple conjunctivitis, three positive cultures were found. The conjunctivitis in the latter patients had no particularly characteristic appearance. Secretion was scanty and of a mucopurulent nature. The bulbar conjunctiva had only moderate vasodilation and subepithelial edema without chemosis. The palpebral conjunctiva was more reddened, comparatively, with a velvety appearance replacing the normally shiny surface. Topical broad-spectrum ophthalmic ointment, solution, or both, cleared these infections promptly as it did in almost all of the other patients of this group; in none was there any evidence of uveitis.

In association with positive PPLO culture, one of the three who displayed positive culture on routine smears and cultures revealed no bacterial findings. However, of the other two, one had *M. aureus* and diphtheroids predominated in the other.

In view of the small number of cases recorded, it is obvious that no great statistical significance (if, indeed, any at all) can be placed on this report. Nevertheless, the review of the pertinent literature and the material presented herein do suggest a relationship of PPLO to uveitis and conjunctivitis that justifies further investigation and evaluation. More comprehensive studies may relate the PPLO etiologically, directly or indirectly (through hypersensitivity), to a number of types of ocular inflammation.

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CULTIVATION OF PLEUROPNEUMONIALIKE ORGANISMS IN REITER'S DISEASE, INCLUDING ONE INSTANCE OF LABORATORY CROSS INFECTION

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The place of pleuropneumonialike organisms (PPLO) in human disease has not been established. During the past twenty years, however, many papers have been devoted to PPLO obtained from human sources. There is still considerable doubt that organisms found so widely in man are pathogenic.[1-4] Many authors have described PPLO found in the human genitourinary tract.[5-14]

In cases of Reiter's disease—a syndrome characterized by conjunctivitis, urethritis, and arthritis—there have been occasional clinical reports suggesting a possible etiological relationship to PPLO.[15-19] PPLO have been found in man in the absence of disease, but generally the literature describes PPLO as present more often in disease than in health.[1-19] In Reiter's disease, a venereal transmission has been suggested.[6,13-15,19]

A previous report of positive PPLO cultures from sexual partners, one of whom had Reiter's disease, was made from our laboratory.[19] The purpose of this paper is to present again the clinical data of that case, in which the organism was exchanged seemingly in what urologists euphemistically termed a "ping-pong" fashion. We also present data on a similar couple and report one instance of probable laboratory cross infection during cultivation of PPLO from a patient with Reiter's syndrome. In addition, data from other patients with Reiter's disease and from various rheumatic diseases will be considered.

Occasionally, during the past 12 years, our laboratory has been called upon to culture conjunctivae, urethral discharges, and joint fluids for PPLO. Our principal interest has been chemotherapeutic trials in rat polyarthritis produced by *Mycoplasma arthritidis* (L4 strain of PPLO). Since ours is not a "service" laboratory and since no attempt was made to culture all rheumatic disease patients in the arthritis clinic, the relative importance of the number of positive cultures to the total rheumatic population in the clinic and our private practice cannot be estimated.

Materials and Methods

The medium used to isolate PPLO was that employed in our laboratory for cultivation of the rat strain (*M. arthritidis*); this medium has been described in detail in other papers.[19,20] Since these studies extended through several years, they were in progress prior to the vogue of employing thallium acetate (1:1000) in the medium as an inhibitor of growth of higher-order organisms. During earlier studies penicillin was added in drops to one portion of the plates used in primary isolation in the fashion described by Melén and Odeblad.[21] This permitted pure subcultures of PPLO from an area of the medium not densely overgrown with other organisms. When commercial media became available for isolation and cultivation (Difco Bacto-PPLO agar and Bacto-PPLO serum fraction), they were employed with thallium acetate 1:1000, rather than penicillin.

Identification of PPLO was made if (1) Wayson- or Giemsa-stained impression preparations of colonies revealed typical spongelike structures; (2) teardrop or fried egg–shaped colonies were observed with the dissecting microscope or low power of the ordinary microscope; (3) the growth on the plates could not be removed with a loop or needle; (4) organisms failed to grow in the absence of their protein requirement; and if (5) filterability was proved, although this was not done in all instances, since the other criteria were regarded as sufficient.

In some instances plates were streaked with the cotton swab on which the original specimen was collected; in others, the swab was swirled in liquid medium, and about 0.5 ml. of this material was streaked on the plate with a sterile pipette. In cases where gross contamination was present, the liquid medium, which had also been incubated, was passed through a Seitz filter, and plates were inoculated with the filtrate. This method has proved satisfactory in several instances in our laboratory; however, it may have been responsible for the contamination that resulted in the technician aquiring acute conjunctivitis and urethritis concomitant with performing Seitz filtrations of positive cultures isolated from Patient 1 (*see below*).

Incubation was at 37° C. No attempt was made to isolate anaerobic forms. Cultures were examined daily after 48 hours, and "blind" subcultures were made in 7 days if the original plates were not positive after that time.

Case Reports

Case 1. A white male, age 46, had been treated for urethral discharge at a city venereal disease clinic. The usual bacteriological and serologic tests were performed and found to be negative. The patient gave a history of previously having had untreated "gonorrhea" 3 times in several years. The disease showed the following chronology.

February 6, 1950. The patient complained of having had a urethral discharge for 4 weeks, bilateral conjunctivitis for 3 weeks, and pain in the right ankle and left wrist and painful stiffness of the neck for 1 week. There was also slight pain in the left inguinal region and some itching of the glans. Examination showed no visible urethral discharge, but the urine from Glass 1 grossly showed large white threadlike strands and, microscopically, the uncentrifuged sediment showed a great many leukocytes; there were 3 erythrocytes per high dry field, and bacilli and Gram-positive cocci were also present. Urine from Glass 2 showed 10 leukocytes and 1 erythrocyte per high dry field. The prostate was symmetrically enlarged to about twice normal size and was soft and nontender. Prostate secretion showed many white cells and occasional erythrocytes, as well as Gram-positive short rods and cocci. The conjunctivae were injected bilaterally and the ophthalmologist diagnosed "nonspecific conjunctivitis." A firm 1.0- × 2.5-cm. left inguinal node and several small right inguinal nodes were palpable.

February 9. The clinical impression was Reiter's disease with secondary bacterial infection. Treatment was begun with sulfadiazine, 1.0 gm. 4 times daily.

February 10. Cultures of the conjunctivae of both eyes and of the urethra were positive for PPLO. The right ankle and left wrist continued to be painful. Administration of Aureomycin (250 mg. 4 times daily for 7 days) was started.

February 17. There was less urethral discharge, and the conjunctivae appeared to be less inflamed. Culture of urine for usual pathogens was negative.

February 21. Exacerbation of arthritis and conjunctivitis appeared 2 days after Aureomycin was discontinued. Culture of urine for usual pathogens was again sterile. Aureomycin was given in the same dosage for 14 more days.

March 6. Urethral discharge had become minimal. The eyes looked grossly normal. Some pain persisted in the right shoulder.

March 10. There was no further joint pain, but urethral discharge still showed a slight drop in the mornings. The right eye and urethral cultures were again positive for PPLO.

April 7. There was no urethral discharge, but the patient reported pus present in the eyes every morning. There had been further stiffness of the neck.

April 10. Aureomycin eye drops were given.

April 21. Eyes appeared to be normal.

June 2. Conjunctivae were clear and cultures for usual pathogens were negative.

Case 2. A 23-year-old white male laboratory technician developed acute conjunctivitis while handling specimens from Case 1. Broth cultures overgrown with bacteria were transferred to a Seitz filter by pouring from the tube. The subject subsequently admitted that droplets of the culture probably splashed on the table top as well as his hands. He developed no joint symptoms, but there was a slight urethral discharge present in the mornings. Smears from his conjunctivae were cultured and found positive for PPLO. The technician was treated promptly with Aureomycin (250 mg. 4 times daily for 3 days). During the treatment the urethral discharge disappeared, and the conjunctivitis apparently subsided. About 4 weeks later, when he was again subculturing in the same manner additional positive PPLO material from the same patient, he again developed acute conjunctivitis that again yielded positive cultures for PPLO and negative cultures for ordinary pathogenic bacteria. The second episode, which could have been an exacerbation of the first, finally subsided on further treatment with Aureomycin (250 mg. 4 times daily for 3 more days).

As a result of this apparent cross infection in the laboratory, the technique was altered so that, when handling human cultures, sterile cotton–plugged pipettes were employed during transferring procedures.

Case 3. A 30-year-old white male was seen on May 19, 1950, complaining of painful joints, purulent conjunctivitis, and urethritis. He had been aware of the conjunctivitis and urethritis since January. All ordinary serologic and bacteriological tests had been negative elsewhere. Diagnosis of Reiter's disease had been made, but he had been given no therapy other than prostatic massage. In February he noted pain for the first time in the hamstring muscles (bilaterally), as well as in both arms. In March the urethral discharge had spontaneously disappeared for 2 weeks and the conjunctivae cleared from time to time. There was no diarrhea. A transient rash had appeared on the fingers. He had had a nongonococcal acute balanitis in 1942, and cultures of the conjunctival and urethral discharge were positive for PPLO. He was started on treatment with Aureomycin (250 mg. 4 times daily) and was told to use Aureomycin eye drops. The subsequent course of the disease had the following chronology.

May 28, 1950. The urethral discharge and the conjunctivitis had subsided. The patient no longer had leg pain, but suffered one episode of arthralgia in the right shoulder. By this time he had also developed a moderate diarrhea, presumably due to the medication.

June 27. The patient still had fleeting pains in the right shoulder and arms. There was no urethral discharge, but there was a slight exudate at the inner canthus of the eyes in the mornings. At this time cultures of the conjunctivae and urine were negative for PPLO.

May 24, 1951. After contact with Patient 4 on an island in the South Sea area, he again developed purulent urethral and conjunctival discharge, together with aching and stiffness of the right hip, knee, ankle, and toes. He administered Aureomycin to himself in a dosage of 1.0 gm. daily for about 2 weeks. During this time all signs and symptoms disappeared.

July 16. Conjunctivae were hyperemic and the prostate was boggy and enlarged. Prostatic secretion contained 5 to 10 leukocytes per high dry field. Unfortunately, at this time we were unable to make a culture for PPLO. It is interesting to note that, following prostatic massage, the knees became painful for several days. Aureomycin (0.5 gm. daily) was resumed, and the patient was given a series of prostatic massages. After each massage the knees became painful for 2 or 3 days.

November. The patient was seen by a urologist, who found the posterior urethra to be sclerotic and scarred about the prostatic ducts, which were hyperemic. From that time he was treated by the urologist and has had no recurrence of Reiter's disease.

Case 4. This 22-year-old white female was first seen by us August 20, 1951, when she complained of purulent vaginal discharge. Her illness had begun in June 1950, when her menses became irregular, occurring every 14 days, the bleeding lasting 8 to 9 days. At this time she was stationed on an island in the South Sea area. Concurrently she became malaised and weak and, in July 1950, a foul-smelling dark vaginal discharge appeared. The chronology of the case was as follows.

January 1951. The patient, then in France, found that symptoms (described above) persisted. A laparotomy was performed. The surgical note described the right ovary as "congested." An oophorectomy and salpingectomy were also performed. In spite of surgery, during which both penicillin and a sulfonamide were infiltrated into the operative site, and in spite of postoperative treatment with parenteral penicillin and the employment of vaginal douches and diathermy, a profuse vaginal discharge persisted. The surgeon was understandably quite perplexed, since he had been unable to culture any pathogenic bacteria from the swabs taken during surgery. His note described extensive fibrous adhesions among the pelvic viscera.

August 20. There was no evidence of conjunctivitis or arthritis. A slight gingivitis was present and body temperature was 37° C. Erythrocyte sedimentation rate was 13 mm./hour (Wintrobe method). Pelvic examination showed some tenderness on movement of the uterine fundus. There was no fixation or adnexal mass. A purulent discharge issued from the cervical *os.* Culture of the discharge was positive for PPLO and negative for *Neisseria gonorrhoeae.* The ordinary culture was positive for *Staphylococcus albus* and *Staph. aureus.* Treatment consisted of Aureomycin (1.0 gm. daily) and zinc sulfate douches daily. Within 3 days there was a diminution in the leukorrhea.

September 11. The discharge had completely disappeared. A vaginal culture at this time was negative for PPLO.

September 4, 1952. The patient had a slight recurrence of the vaginal discharge, but this subsided promptly on treatment with Aureomycin. There has been no further difficulty.

Examination of the dates of contact between Patient 3 and Patient 4 would appear to indicate that the male subject was the original bearer of the disease. He had had acute balanitis in 1942 and his symptoms began in January 1950, while the symptoms of the female subject appeared first in June 1950. Following what appeared to have been adequate therapy with Aureomycin, Patient 3 returned to contact with Patient 4 and promptly suffered recurrence of Reiter's disease. It is probable that she was harboring the organism at that time, since she had had no treatment with tetracycline medication. She did not, however, develop a typical Reiter's syndrome—one that rarely has been described in the female.[13]

Case 5. A 29-year-old white male was first seen on June 10, 1954, complaining of multiple joint aches and some stiffness of the back since the spring of 1953. Although he had had some fleeting pains in the proximal interphalangeal joints of his fingers in adolescence, he had had no articular troubles in the interim. In April 1951 he had had acute prostatitis and seminal vesiculitis, and took large amounts of antibiotics at that time. Routine cultures, done elsewhere, showed *Escherichia coli*, *Staph. albus*, and Gram-positive diplococci. Six months later he had a persistent diarrhea with large foamy stools that contained blood. Proctoscopic examination showed "injected friable mucosa in the rectum and sigmoid colon." In June and July 1954 cultures of the urine were positive for PPLO in our laboratory. In June the patient began taking 50 mg. per day of terramycin in a single dose.[17] During the first few days the urethral discharge disappeared, but then returned while he was still taking the minute dose of the antibiotic. The patient became frightened of further antibiotic therapy after it became clear that small dosage apparently inflamed the colitis. In September 1954 he had some dermatitis with pruritis and fissuring on the scrotum and the penis. October 16, 1954, another urine culture was positive for PPLO and the joint pains continued to bother him. Nine days later a culture from the conjunctivae was also positive for PPLO, and he had a conjunctivitis with purulent strands in the mornings. The patient was treated with 0.5 gm. of Dystricin (a mixture of equal parts of streptomycin and dihydrostreptomycin) intramuscularly daily for 10 days. Early in 1955 he developed shallow ulcerations on his nasal septum, cultures of which were also positive for PPLO. Between this time and January 1956 he continued to have recurrent conjunctivitis, seminal vesiculitis, colitis, bronchitis, arthritis, and mucosal lesions in the nose. On January 31 and February 24, 1956, cultures of the urine, sputum, and cough plate were all positive for PPLO. In February

1956, the patient's sexual partner (Case 6) who had had "recurrent cystitis and cervicitis for 5 years" was examined. Cultures of her vagina, nose, sputum, and urine were positive for PPLO. The patient began medication with cysteine-HCl (250 mg. 4 times per day), and cultures similar to those of February 24 were taken on March 9, March 26, and April 17, 1956; all were negative. He was tried on amino acid since he was unable to tolerate the tetracyclines and because of the suggestion in our work with rat arthritis (due to PPLO) that addition of cysteine-HCl had a beneficial effect. On August 3, 1956, he had a purulent conjuntivitis, fever, and an ulcerated area on the tongue. These promptly subsided. The patient has been seen many times since then—last on November 25, 1958—and has no more signs or symptoms suggestive of Reiter's disease.

Case 6. A 35-year-old white female was seen on February 9, 1956 at the request of Patient 5 because of a 5-year history of recurrent cystitis and cervicitis. At that time she also had an upper respiratory infection and a cough. Cultures from her vagina, nose, sputum, and urine were positive for PPLO. The usual cultures showed "typical nasopharyngeal flora." The patient was treated with terramycin in dosages of 250 mg. 4 times daily for 4 days, followed by 250 mg. 3 times daily for 3 days. She has experienced no further recurrence of her multiple evidence of infection.

In considering Cases 5 and 6 together it is noteworthy that here again, as in Cases 3 and 4, the male had the arthritic symptoms and, following treatment of the female sexual partner, had no further symptomatology suggestive of Reiter's disease. The exacerbation of arthritic symptoms in Case 3 following prostatic massage is also of interest.

Case 7. A 26-year-old white housewife was seen on April 10, 1955, when 3 months pregnant. She complained of having had painful, swollen, stiff fingers for 4 months. She had had "false positive" serologic tests for syphilis 2 years previously. The *Treponema pallidum* immobilization test was negative. In April 1955 she again had "false positive" serology, a clinical picture resembling rheumatoid arthritis, a positive L.E. cell preparation, transient rash on the forearms, and a positive vaginal culture for PPLO. Because of the pregnancy, strong medications in large dosage were avoided and she was given only 50 mg. of terramycin per day. As the pregnancy progressed and the medication continued, the arthritic symptoms subsided. On October 7, 1955, she delivered a baby girl. Cultures of urine and prostatic fluid from her husband were negative at this time, and a culture of the urethra of the infant was also negative for PPLO. On January 24, 1956, she was again seen regarding multiple joint pains. She had discontinued the Terramycin 2 weeks previously. Following resumption of medication, she continued to do well. She was last seen in April 1956, when she had several tiny crusted skin lesions on the forehead, but no joint symptoms. She now lives elsewhere and, in April 1957, had recurrent joint pains.

PPLO was cultured from 2 more males with Reiter's disease; it was not possible to study their sexual contacts. In another male with Reiter's disease we were unable to culture PPLO from the urethra, but he had taken Aureomycin for 2 weeks before we examined him.

From time to time we cultured vaginal discharge, conjunctival exudate and, occasionally, joint fluid from patients who appeared to have evidence of "infection" in the presence of typical rheumatoid arthritis. Twenty-one of 25 such instances resulted in positive cultures of PPLO. Likewise, when infection was suspected we obtained positive cultures in 4 of 5 patients with ankylosing spondylitis. On the other hand, when an attempt was made to culture PPLO from the cervix in 20 women with rheumatoid arthritis where there was no gross evidence of cervicitis or of vaginal discharge, all cultures were negative. We did not attempt serologic studies to reveal the possible presence of antibodies. All of the 20 rheumatoid arthritic women with the negative cultures were "old" cases under treatment of various kinds.

Summary

(1) While working with PPLO cultures from a male patient with Reiter's disease, a young male technician developed acute urethritis and conjunctivitis from which PPLO were recovered.

(2) Both of 2 males with "recurrent" Reiter's disease, from whom positive PPLO cultures were obtained, recovered when their sexual partners were treated with tetracyclines after they were found to have positive vaginal cultures of PPLO.

(3) PPLO were cultured from 2 other patients with Reiter's disease where it was not possible to examine their sexual partners. In one male with Reiter's disease who had already been treated with Aureomycin it was not possible to recover PPLO from the urethra.

(4) PPLO were recovered from vaginal culture in a young pregnant woman with probable systemic lupus erythematosus; subsequently, she did well symptomatically as long as she took 50 mg. of Terramycin daily.

(5) PPLO were also recovered from 21 of 25 patients with rheumatoid arthritis and from 4 of 5 with ankylosing spondylitis where there was some evidence of infection present. Cultures were made from the uterine cervix, vagina, urine, conjunctivae, sputum, and skin lesions. In *none* of 20 women with typical rheumatoid arthritis who were already under treatment and selected at random from a large group were we able to recover PPLO.

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PLEUROPNEUMONIALIKE ORGANISMS AND L FORMS OF BACTERIA

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Have pleuropneumonialike organisms (PPLO) a bacterial origin or do they constitute an independent group? Dienes and Weinberger[1] and Tulasne[2] think that the similarities between PPLO and L forms of bacteria are sufficient to justify their parentage. However, most other authors, employing morphologic, metabolic, or experimental arguments, dismiss any idea of a relationship between PPLO and bacteria.

Since Dienes[3] isolated PPLO in an infection of a female Bartholin's gland, many studies have shown the frequent presence of these organisms in the genital tracts of both males and females. Several workers noticed the appearance of bacteria of the corynebacteria group in PPLO cultures. Taking these results into consideration, it is, of course, necessary to exclude any possibility of laboratory contamination resulting from PPLO isolation techniques and allowing a passive transfer of bacteria in bacteriostasis.

One of us (R.M.)[4] isolated corynebacteria in PPLO cultures and showed antigenic relations between this organism and the corresponding PPLO. Dienes (personal communication) and Peoples *et al.*,[5] later recorded similar observations, and Wittler *et al.*[6] cultivated in tissue cultures a PPLO strain that, after showing morphologic changes, gave in culture a corynebacterium with the same biochemical properties and the same antigenic structure as the PPLO. Finally, Smith *et al.*[7] demonstrated statistically that corynebacteria appearing in PPLO cultures are not contaminants. The isolated organisms are different from the airborne and oral corynebacteria and show antigenic similarity to the corresponding PPLO.

We have studied 7 strains of human genital PPLO (several isolated in this laboratory for many years) and, for comparison, a strain of pleuropneumonia (*Mycoplasma mycoides*) and a strain of agalactia (*M. agalactiae*) recently isolated. All were previously transferred at least 10 times on agar medium without antibiotics. We isolated corynebacteria from three of the PPLO strains, one of them subcultured for 6 years. We have never found bacteria either in pleuropneumonia or in agalactia cultures that were handled in exactly the same manner. We, too, noticed once more the unexpected appearance of corynebacteria in PPLO cultures. Other authors have pointed out the appearance of several bacteria in animal PPLO cultures, yet no one has brought forth the conclusive experiment of transforming the bacteria into PPLO. The similarities between PPLO and L forms and the above-reported observations cannot be dismissed. Many authors, however, deny any relation between L forms of bacteria and PPLO.

Years ago the reversion of the L forms was cited as arguing against a relation with PPLO. However, fixed L forms that do not reverse were obtained. The main arguments are based essentially on morphologic and metabolic grounds. We cannot study all the differences of morphologic order described;

hey do not seem vital to us, for we must allow for the plasticity of the L forms as well as of the PPLO. According to such factors as the richness and the wetness of the medium and the culture density, these organisms present essential modifications in the microscopic and macroscopic aspect.

The arguments of metabolic orders are more important. Although several L forms and PPLO require serum for cultivation, it seems that growth factors differ. Edward and Fitzgerald[8] and Smith and Lynn[9] showed the essential role of cholesterol in PPLO growth. Although knowledge about L-form metabolism is still rudimentary, all agree that cholesterol has no effect. The work of Kandler *et al.*,[10] grounded on the action of some respiratory poisons, shows that L-form and bacterial metabolisms are comparable, whereas the studied PPLO seem to be provided with enzymatic systems different from the bacterial ones. It must be pointed out, however, that most of the studies were done with some bacterial L forms easily available, particularly with the *Proteus* L form.

Edward and Fitzgerald[11] pointed out the specific inhibiting action of antisera (without complement) on cultures of human genital PPLO. Many bacteriologists since have used this property to determine the serologic type of PPLO. Edward and Fitzgerald obtained inconsistent results in reproducing the same experiences with L forms of bacteria, and von Prittwitz-Gaffron[12] observed no inhibiting action on L cultures by the O and H antibacterial serum, even with complement.

To us, this question seemed worthy of restudy. We chose 3A and 3B L forms of *Proteus* P18. Anti-*Proteus* and anti-3A L-form rabbit sera were used. The agglutinin titer against O, H, and L antigens was studied. These sera were also saturated with the O and H bacterial antigens and the L form. The inhibiting action of each serum was studied by putting into the agar medium paper disks impregnated with 0.2 ml. of pure or diluted serum. The medium was inoculated with the L forms after 36 hours at room temperature. After it was incubated for 5 days, we measured the diameter of the inhibition area. Controls were included with rabbit serum, taken before immunization, and with heterologous sera. TABLE 1 summarizes our results.

None of the control sera showed any inhibiting action on the L-form culture. With none of the sera was 3B L-culture inhibition observed. Thus we can say that, as PPLO, the 3A L form is inhibited in culture by specific antiserum; only the antibodies corresponding to the O bacterial fraction and to the L form gave positive results. Antibodies corresponding to the H antigen are inactive. We showed, as have other investigators, that L forms are worse antigens than are bacterial antigens. Moreover, in one case, the saturation of an anti-L serum by bacteria brought the disappearance of the agglutinins, without complete abolition of the inhibiting power on the culture. Instead of considering the possibility that there are 2 different antibodies, we thought it probable that the less precise method of agglutinin study was the source of the difficulty. The different response of 3A and 3B L forms to the antiserum strengthened other biological discrepancies (reversion ability and possibility of cultivating into synthetic medium) and indicates that these 2 L types are not comparable. Other experiences, which we cannot report here, showed that L forms obtained on hypertonic media, even when they lost their reversion ability, were not inhibited by the antiserum and responded as 3B type.

In what manner does the serum act on the 3A L form? Taubeneck and Böhm[13] recently showed, with a *Proteus*, that only the 3B L forms, as bacteria, are susceptible to phage. Alterations of the cell-limiting membrane occurring during transformation from the 3B into the 3A type are certainly followed by the loss of phage-adsorption possibility and reversing ability, and by the appearance of a sensitivity against specific antiserum in culture.

The review of the literature and the observations reported in this paper show that results very different and often quite contradictory have been observed on the same point by many investigators who have worked on L forms of bacteria. We think that these divergences can be explained by the lack of unanimity among these workers about what must be called L forms. Some will designate as L forms only the 3A colonies of Dienes; others, the 3A *and* 3B colonies; another again, the gigantic forms described by Tulasne[14] in *Vibrio* species, or the elements found on hypertonic media; and another, the protoplast, or every round deformation seen in bacteria under the microscope. However, all these organisms have very different biological properties, and it is not surprising that the recorded results often seem to conflict.

TABLE 1

Antiserum	Saturated with	Agglutinin rate			Inhibition of the 3A L cultures* Serum dilutions					Inhibition of the 3B L cultures Serum diluted 1/2†
		H	O	L	1/2	1/5	1/10	1/20	1/40	
Proteus O-H (No. 27)		1/6400	1/1600	1/800	36	31	30	26	24	0
Proteus O-H (No. 25)		1/800	1/800	1/800	38	30	27	27	25	0
Proteus O-H (No. 26)		1/800	1/600	1/800	40	31	29	25	22	0
L form (No. 58)		0	1/100	1/150	29	21	19	0	0	0
L form (No. 59)		0	1/100	1/100	28	27	22	20	0	0
L form (No. 60)		0	1/200	1/100	30	21	0	0	0	0
L form (No. 63)		0	1/320	1/160	30	25	22	20	0	0
Proteus O-H (No. 27)	*Proteus* O	1/600	0	0	0	0	0	0	0	0
Proteus O-H (No. 27)	L form	1/3200	0	0	25	0	0	0	0	0
L form (No. 59)	L form		0	0	0	0	0	0	0	0
L form (No. 59)	*Proteus* O-H		0	0	28	22	21	0	0	0

* Inhibition area is given in millimeters.
† Identical results obtained with dilutions of 1/5, 1/10, 1/20, and 1/40.

This incomplete study of PPLO and 3A L forms prevents concluding in favor of the identity of these two types of organisms. On the other hand, there is no conclusive evidence for dismissing a relationship between PPLO and the L forms of bacteria.

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DISCUSSION OF PART VI

D. G. FF. EDWARD (*Wellcome Research Laboratories, Beckenham, Kent, England*): Ever since the first isolation of organisms of the pleuropneumonia group from the human genital tract, great interest has been taken in their relationship to infection. There would seem to be little doubt that the organisms can be pathogenic for man. Their pathogenicity would appear to be similar to that of *Escherichia coli*, which is a normal inhabitant of the gut, yet can produce a pyogenic infection if it gains access to other parts of the body. There seems little doubt that organisms of the pleuropneumonia group can cause puerperal fever, salpingitis, and abscesses in the neighborhood of the genital tract. However, in regard to pathogenicity the main question at issue is whether the organisms are a cause of nongonococcal urethritis. This disease is of great importance and its etiology remains uncertain. Since these organisms are present in a proportion of cases it is tempting to suggest that they cause the infection.

Many investigators have studied the relationship of the organisms to nongonococcal urethritis; in various surveys the proportion of positive isolations from cases has varied, but has never exceeded 50 per cent. On the other hand, similar organisms have been isolated from healthy individuals examined as controls. There are differences of opinion as to what constitutes a control group, and doubts have been expressed as to whether the genital tracts of all the controls were completely healthy. Klieneberger-Nobel has examined boys before puberty, but I regard as a control group men from the general population who are not attending a venereal disease clinic. I consider that it is highly significant that a high proportion of these men have *Mycoplasma hominis* in the urethra. On the other hand the organism can be isolated only from one half or less of the cases of nongonococcal urethritis, although it is not a difficult organism to isolate. There is no difference, clinically or in response to treatment, between cases with positive isolations and those in whom the organism cannot be demonstrated.

These findings suggest strongly that *M. hominis* is not the cause of nongonococcal urethritis. It would appear more likely that it is a commensal, although it is impossible to exclude the possibility that it may produce some ill effects when it gains access to the urethra. It would seem that little can be gained from a repetition of the many surveys already carried out in which the organisms have been sought in diseased and healthy individuals. If the question of pathogenicity is to be decided a new approach must be made.

E. A. FREUNDT (*Statens Seruminstitut, Copenhagen, Denmark*): I do not think that the different incidence of PPLO in the male urethra found by Klieneberger-Nobel and by me can be explained by different methods of procuring specimens for cultivation. Actually, the technique I used would not seem to differ essentially from that of Klieneberger-Nobel. My specimens were not taken merely from the "external surface" of the urethral meatus, as supposed by Klieneberger-Nobel, but from the anterior urethra by means of cotton-wool swabs. These were inserted as deep as possible into the urethra after cleaning the meatus with saline. Moreover, I don't think that the microbial flora of the ower few centimeters of the anterior urethra differs very much from that of he urethral orifice.

Recently, in our laboratories, Scheibel and Assandri conducted experiments imilar to those of Rubio-Huertos. They obtained stable L forms from 4 strains f *Clostridium tetani*. These remained stable during about 20 transfers. These -phase variants were induced by penicillin. Scheibel and Assandri found hat 3 of those strains were still pathogenic and that their toxicity titer was lmost as high as, or perhaps a little higher than, that of the bacillary phase.

The fourth strain behaved differently, since only 1 of 4 L colonies was found o be pathogenic, and this too began to be nontoxic after a few transfers. The train differed from the other strains in that it was nonsporulating. It may e important to note that this strain was obtained from the Harvard Medical chool, Boston, Mass. I wonder if this strain is identical with that investigated by Dienes some years ago, a strain that is also found to be nontoxigenic n its L phase.

Louis Dienes (*Massachusetts General Hospital, Boston, Mass.*): It is difficult o decide from the photographs and the microphotographs whether the pleomorphic forms studied by Doctor Rubio-Huertos are really L forms. Glycine, which was present in the media, produces similar pleomorphic forms from acteria. The small granules appear more like bacteriophages than L forms. t is more likely that the structures observed in this study were not L forms.

E. M. Lerner (*National Institutes of Health, Public Health Service, Bethesda, Md.*): Rubio-Huertos made an interesting point: L forms do appear to be more athogenic than the normal form. In the past it has been our experience with *Clostridium tetani* that the appearance of toxin in the culture medium is accompanied by dissolution or lysis of the cell wall. A number of manipulations an be carried out that change the growth characteristics, the iron requirements, r the fermentative characteristics, but toxin production is eliminated almost mmediately. These L forms continue to produce toxin.

Dorothy M. Powelson (*Purdue University, Lafayette, Ind.*): As a bacterial ytologist, I cannot help but challenge Rubio-Huertos in regard to these small granules which, as I understand him to say, are viable: that is, will reproduce the species. I think these small granules are very similar to those often termed metabolic granules. I should like to know whether any work has been done to establish the fact that these granules are really likely to be reproductive units. It seems to me that one way to approach this would be to demonstrate that these granules do contain DNA, and not RNA, which would be typical of the metabolic granules.

M. Rubio-Huertos (*Instituto Jaime Ferrán de Microbiologia, Madrid, Spain*): I assumed that everyone was familiar with the L cycle described in 1953 by Tulasne, who demonstrated the passage from the normal bacillary form to large bodies and then from the large bodies to the very small units. Tulasne proved that these small units were composed of desoxyribonucleic acid, as well as ribonucleic acid.

K. Liebermeister also has demonstrated the L cycle, that is, the formation from the normal form to large bodies with intermediate forms and also the formation of filterable forms. An organism in the L form must be proved to pass all of these cycles. An organism cannot be called an L form if it is only a globular form or a colony. A filtration of a small granule must be made and the original bacteria recovered from it. This is the only proof.

Ruth G. Wittler (*Walter Reed Army Medical Center, Washington, D.C.*): PPLO represent the type of agent that I found in Reiter's disease and was transmissible but not culturable on ordinary agar. I wonder if at times we may not be dealing with an agent that is not yet ready to be cultured on agar.

J. T. Sharp (*Harvard Medical School, Boston, Mass.*): Among those of us who see patients with Reiter's syndrome clinically, I think there is very little agreement as to response to antibiotics and, in my experience, most patients with Reiter's syndrome do not respond clearly to any known antibiotic.

William C. Kuzell (*Stanford University School of Medicine, San Francisco, Calif.*): I think that Sharp's comment is apt. I had a similar experience in the patients with Reiter's disease whom I followed. When I speak of the patients as being improved with antibiotics I refer simply to our inability to culture further the PPLO. The symptoms and signs of the disease continue and seem to pursue their usual well-known clinical course.

E. A. Freundt: I wonder whether Kuzell classified as species the organisms he cultivated, and how they were identified. A few years ago I obtained a few strains from Canada that then were claimed to be isolated from cases of polyarthritis, and they were surely not PPLO.

William C. Kuzell: Identification was based upon the following criteria: (1) Wayson- or Giemsa-stained impression preparation of colonies revealed typical spongelike structure; (2) characteristic teardrop or fried egg-shaped colonies were observed with the dissecting microscope or the low power of the ordinary light microscope; (3) the growth on the plates could not be removed with a loop or a needle; (4) the organisms failed to grow in the absence of their protein requirement; and (5) filterability was proved.

OBSERVATIONS ON THE TYPE SPECIES *MYCOPLASMA MYCOIDES*

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It is just sixty years since Nocard and his associates (1898) first demontrated and cultivated the bovine pleuropneumonia organism, although the disease in cattle was first recorded as long ago as 1693 (Henning, 1948). The ntroduction of improved staining methods and dark-field illumination established the fact that the organism was very variable in morphology, ranging rom minute filter-passing spheroids to long filamentous branching processes, and that it underwent a complex life cycle. Because of the radiation of filaments from the round forms, the name *Asterococcus mycoides* was commonly used for many years. More recently, the suggestion of Edward and Freundt 1956) that the name *Mycoplasma mycoides* should be adopted has gained considerable acceptance.

M. mycoides is the causal agent of an acute, subacute, or chronic disease of cattle only, characterized by pneumonia, a serofibrinous pleurisy, and edema of the interlobular septa of the lungs. The disease occurs in Africa, Asia, and Australia and is the cause of serious economic loss. Bovine pleuropneumonia n epidemic form is probably more difficult to control than any other cattle disease of major importance. Although the mortality is relatively low, the morbidity is high, and many apparently fully recovered animals continue to harbor the organism in sequestered lesions in their lungs. These "carrier" animals are responsible for the spread and perpetuation of the disease which, n consequence, is extremely difficult to confine and control, particularly in the vast underdeveloped areas where it is now commonly found. Under such conditions prompt diagnosis and the speedy application of regulatory measures such as slaughter, the imposition of quarantine, and mass immunization campaigns are frequently impossible. Long before effective action can be taken, the disease has spread to neighboring herds and other areas. Preventive immunization on a massive scale would be the best means of control, but a really efficient and safe vaccine capable of stimulating an enduring immunity of high quality and sufficiently stable to withstand the adverse effects of hot climates and poor communications has not yet been elaborated.

M. mycoides is the type species of the PPLO or mycoplasma group of organisms. It is extremely pleomorphic, and minute elementary bodies, rings, branching filamentous forms, and various bizarre elements may all be seen by appropriate methods of examination. It is generally considered that the first of these elements represents the infective particle. The organism can be cultured readily in various types of nutrient broth containing serum, one of the best being tryptose broth containing yeast extract and pig serum (Newing, 1956). Edward and Fitzgerald (1951) have shown that serum can be replaced by an ethereal extract of egg yolk and that cholesterol is an important growth-promoting factor. The findings of Rodwell (1956) suggest that the principal function of serum is to provide a heat-stable protein fraction, cholesterol, and oleic acid as a nontoxic complex. Aeration, which can be achieved conveniently by the use of magnetic stirrers, encourages a more profuse growth. During the logarithmic phase of growth in fluid cultures, usually about 48 hours after inoculation, characteristic long filaments commonly referred to as "comets" may be seen with the naked eye. On solid media containing serum, minute dewdrop colonies develop slowly during a period of 5 to 6 days and grow down into the substance of the medium.

The complex life cycle of *M. mycoides* has been studied by a number of workers, notably Turner (1935), Tang *et al.* (1935), Wilson and Miles (1945), Sabin (1941), Klieneberger and Smiles (1942), and Klieneberger (1942). Broadly speaking, two methods of multiplication have been described—that is, by segmentation followed by the development of elementary bodies within a limiting membrane, and by the development of buds and branching filaments in which elementary bodies are formed by protoplasmic condensation.

The antigenic structure of *M. mycoides* is also complex, and at least three antigenic components have already been demonstrated by chemical fractionation (Dafaalla, 1957), serologic tests (Priestley, 1957), and gel diffusion (White, 1958). There is no evidence, however, for the existence of more than one antigenic type.

Cultures suitably concentrated by centrifugation may be used for complement fixation (CF), agglutination, and precipitation tests. The latter has been developed recently by White (1958), using the gel double diffusion precipitation technique. When such materials as infected lung, gland, and pleural exudate are opposed to hyperimmune serum prepared by inoculating rabbits with washed organisms, three distinct lines of precipitation develop, indicating a complexity of antigenic structure. This promises to provide a rapid means of diagnosis, particularly as positive reactions can still be obtained with putrified material or material preserved for long periods with formalin. This test may also prove invaluable in helping to classify the various species and types within the group *Mycoplasma*. The quantitative assessment of antigen and antibody content, however, is much more difficult.

The property of agglutination has been utilized by Newing and Field (1953) and Newing (1955) in the development of a rapid slide agglutination test. Because it uses stained antigen and whole blood, this test is particularly suitable for screening large numbers of cattle in the field, especially in circumstances where it may take several days for samples to reach the nearest diagnostic laboratory. The test is not foolproof, however, and batches of antigen vary in sensitivity, so that false positives sometimes occur. Positive sera, however, can be confirmed by the more reliable CF test.

Despite some drawbacks, the CF test, first elaborated by Campbell and Turner (1936), is still the most reliable method of identifying infection and the carrier state. Since sera from animals in the incubative stages of the disease may be negative, repeated tests at intervals of a week or more may be necessary to recognize infection. It should be emphasized, however, that the presence of complement-fixing antibodies is not necessarily synonymous with immunity, nor does their absence mean that an animal is susceptible. It is not an uncommon experience to find animals positive to the test, yet reactive to challenge, while others, whose blood is negative, prove to be fully resistant.

Under natural conditions the disease caused by *M. mycoides* is directly contagious, and transmission occurs by the inhalation of organisms expelled by infected animals. The organism is specific to cattle, and there is no evidence of an intermediate host. A most serious and characteristic aspect of bovine pleuropneumonia is the existence of carrier animals or "lungers," which appear clinically healthy, but in fact have localized infections in their lungs. These foci are surrounded by fibrous tissue in the form of a sequestrum that, in time (perhaps after many months), may break down. Still viable organisms are thereby liberated into the bronchi and may then infect susceptible animals that are in contact.

Experimental transmission is surprisingly difficult and may be achieved with regularity only by exposing susceptible cattle to infective aerosols in closed chambers. The parenteral inoculation of virulent cultures causes a flat, plaquelike swelling that may spread down the side of the body, between the limbs, and along the abdomen. The subcutaneous tissues are grossly thickened and made edematous by the presence of quantities of a straw-colored fluid commonly known as "tumor lymph" and containing large numbers of organisms. Death occurs in about 50 per cent of such cases.

This syndrome is quite unlike classic bovine pleuropneumonia, in which the pathological changes are found in the lungs. Lesions are commonly confined to one lung, with adhesions of the affected parts to the chest wall by a yellow mass of spongy tissue. A fibrinous pleurisy, edema of the mediastinum, and the presence of straw-colored exudate are common findings. Pneumonic areas, small or large, in various stages of hepatization and consolidation are found; these present a typically marbled appearance. The interlobular septa become grossly thickened, and their lymph spaces become distended with fluid, causing a beaded appearance.

Experimental transmission to embryonated eggs and laboratory animals, including the rabbit, guinea pig, golden hamster, and mouse, has been achieved by a number of workers (Hyslop, 1958).

The successful prevention and control of bovine pleuropneumonia is dependent upon rapid diagnosis, restriction of movement, the identification and slaughter of carriers, and the protection of susceptible animals by vaccination. The most urgent single need is for a reliable effective vaccine.

Despite a hundred years of earnest investigations by many workers, attempts to elaborate a vaccine that is at once safe and effective have been largely disappointing. This must be due, in part, to the biological response of *M. mycoides* to artificial culture. While attenuation can be achieved readily by serial passage through serum broth cultures or the fertile egg, experience has shown that the best chance of creating a solid and durable immunity lies in the use of vaccines containing virulent or near-virulent organisms. That this can be done without killing a high percentage of animals is a result of the fact that the parenteral inoculation of virulent cultures does not cause bovine pleuropneumonia. If the virulent inoculum is placed in the tip of the tail, multiplication and spread of the organism is sufficiently slow in most cases for the animal to mobilize an effective defense, with subsequent immunity. This method of vaccination has been used in Australia for more than twenty years with notable success for, although local tail reactions often may be severe, mortalities due to vaccination are usually less than 1 per cent. On the other hand, this method has not found general acceptance in other countries, such as Africa, where until recent years reliance has been placed on repeated inoculations of attenuated cultures. Unfortunately, the attenuation "gradient" of *M. mycoides* is somewhat unpredictable and, not infrequently, batches of vaccine that the makers had every right to believe safe have caused serious reactions and mortalities.

Both the above methods involve the use of wet cultures, the effective life of which is only a few weeks. When attenuated wet cultures are used, the chief difficulty lies in determining the point in subculturing when the vaccine can be considered both effective and safe. Since it cannot be titrated before issue, the potentiality of any given batch cannot be judged until after it has been used in the field, a dangerous and unscientific procedure. Furthermore, there is a growing belief that, in the case of *M. mycoides*, attenuation and ability to immunize are incompatible, and that only virulent or semivirulent cultures will confer adequate protection. For this reason workers in the Sudan (Priestley and Dafaalla, 1957) have experimented with the possibility of using dried cultures of moderately virulent organisms reconstituted in the field with molten agar and inoculated into the tip of the tail. The agar has an adjuvant effect on dried organisms and also helps to localize them. Laboratory trials were encouraging, but the method broke down when used on a field scale.

A new approach was made by Sheriff and Piercy (1952, 1953) and Piercy and Knight (1956, 1957, 1958) when *M. mycoides* was propagated by serial passage in the fertile egg. Attenuation to the point of safety was soon achieved. The great advantage of this vaccine is that it can be dried and stored so that titrations in serum broth and cattle can be carried out before issue. The stability and keeping properties are excellent. However, although many reports from the territories in East and West Africa, where avianized pleuropneumonia vaccine has been used, have been most encouraging, it appears that the vaccine may be too attenuated to withstand the challenge of virulent outbreaks. Attempts have been made, therefore, to raise the efficacy of the vaccine by using organisms at a lower egg passage level but, when this was done, a mortality of 3 per cent resulted. Whether a level of attenuation between the 2 extremes can be found remains to be seen.

Statistical examinations of attenuated avianized vaccine have shown that a significant proportion of animals are immunized against challenge with virulent cultures (Piercy and Knight, 1958). The reason that some remain unimmunized has been the subject of much thought. About 40 per cent of normal animals possess a degree of natural resistance that enables them to recover from the inoculation of virulent cultures. The existence of this semiresistant group may well explain the failure of attenuated vaccines to immunize the whole of a population for, in such animals, the attenuated organisms may be unable to propagate satisfactorily. The immune state of this group, therefore, is not reinforced and is insufficient to withstand a laboratory challenge or severe natural exposure. Experiments were conducted in which vaccination with fully attenuated cultures was followed one month later by the inoculation of semivirulent organisms. It was hoped in this way first to immunize the fully susceptible animals and to reinforce with the second inoculation the immunity of those of the semiresistant group. Results were disappointing in that the esistance to subsequent challenge of the group of cattle receiving the two noculations proved no higher than that of another group receiving one inoculation only of attenuated vaccine.

The position today is that there is a reluctant acceptance in many quarters of the principle that the only way to create solid immunity against bovine pleuropneumonia is by using virulent or semivirulent organisms, for all attempts to elaborate attenuated vaccines have met with only partial success. In countries such as Australia, inhabited by an informed European population, the use of potentially dangerous virulent material has been accepted as a permissible calculated risk with good results. A disadvantage to the present Australian method, however, is the use of wet cultures, and the nearest approach to an efficient and stable product would seem to be a dried avianized vaccine containing relatively virulent organisms. It would be necessary to couple such an approach with a vigorous program of education; one designed to persuade less enlightened populations of stock owners, such as are found in many parts of Africa, that at present, to obtain a solid and durable immunity, vaccines must be used that may cause some undesirable reactions and some mortality.

Addendum

Since this article was written an important monograph, *The Mycoplasmataceae*, by E. A. Freundt (1958), has become available; it records many valuable observations on the morphology, biology, and taxonomy of the PPLO.

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PORCINE POLYSEROSITIS WITH ARTHRITIS: ISOLATION OF A FASTIDIOUS PLEUROPNEUMONIALIKE ORGANISM AND *HEMOPHILUS INFLUENZAE SUIS**

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This study stemmed from observation of a disease affecting piglets that were used in nutritional experiments. Because of the nature of the experiments (fat studies), it was necessary to use, as experimental animals, piglets free of colostrum. These pigs were caught at birth and placed in individual cages in an isolation unit. Within 12 hours of birth, 10 to 15 ml. of 10 per cent porcine gamma globulin was administered intraperitoneally in order partially to replace immune bodies usually obtained from colostrum. At the end of 6 to 8 weeks, these colostrum-free pigs had outgrown their original cages and, in order to continue the experiment, space limitations made it necessary to move them from isolation to a pig farm. After approximately 4 days at the pig farm, the following symptoms of disease were observed: anorexia, high fever, weakness in hindquarters, unsteady gait, and swollen joints. Within a week after the first appearance of symptoms, approximately 70 per cent of the pigs died. Post-mortem examinations consistently revealed fibrinous peritonitis, pleuritis, pericarditis, and excess and viscous synovial fluid in the joint cavities. In addition to the polyserositis with arthritis, there was observed a hemorrhagic gastroenteritis and cystitis. The isolation and identification of infectious agents capable of reproducing this syndrome are the subjects of this report.

Experimental

Essentially, my colleagues and I were interested in identifying a causative agent or agents isolated from diseased colostrum-free pigs on the farm (field cases) and in reproducing the syndrome, as seen in the field, in colostrum-free pigs raised and infected in isolation. Initially, it was not possible to identify bacteria in this syndrome by culturing the liver and spleen from diseased pigs in thioglycollate broth, on blood plates, or by staining with Giemsa impression smears of the liver and spleen; nor was it possible to isolate pleuropneumonialike organisms (PPLO) on heart infusion agar supplemented with 1 per cent Bacto-PPLO serum fraction. However, from 2 different cases an agent was isolated from emulsions of liver, spleen, and joint fluid inoculated into the yolk sac of 7-day-old chick embryos. The emulsions had been treated with penicillin (200 U./ml.) prior to injecting the embryos.

In order to obtain leads regarding the identity of the agent isolated in chick embryos, characteristics of this agent were determined in the developing chick embryo and by electron microscopy. Pooled frozen fluids harvested from the eleventh egg passage served as a source of infectious material for determining the chick embryo characteristics. Techniques were similar in detail to those previously described for characterizing the tendovaginitis agent.[1] Specimens or the electron microscope were prepared by using the agar-drying and flota-ion method.[2]

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Characteristics of the polyserositis agent were found to be as follows:

(1) The agent could be passed through a sintered-glass bacterium-retaining ilter. Before filtering, 0.25 ml. of infectious allantoic-amniotic fluid killed 00 per cent of the embryos at a dilution of 10^5 and, after filtering, it killed 63 er cent of the embryos at this dilution.

(2) Titrations in the developing chick embryo indicated that the agent was nost sensitive to the broad-spectrum antibiotics (Terramycin, Aureomycin, hloromycetin), partially sensitive to streptomycin, and resistant to penicillin.

(3) The yolk sac route of infecting embryos was the preferred route of noculation, killing faster than the amniotic route. The allantoic route was he poorest. Generally, 100 per cent of the embryos were killed in 4 to 12 lays (most on the fifth to eighth day) after infection via the yolk sac of 7-day-ld embryos.

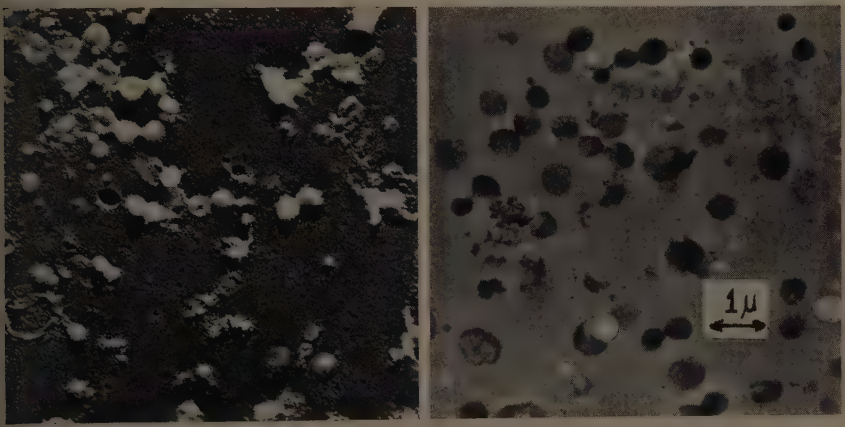

FIGURE 1. Electron micrographs of pig polyserositis agent. Examinations were made vith an R.C.A. electron microscope. Infectious allantoic-amniotic fluid, differentially centri-uged, served as a source of material. Electron micrograph on left, shadowed; on the right, inshadowed. ×4000.

(4) Morphologically, as seen with the electron microscope, the agent ap-eared to be a highly plastic coccobacillus ranging in size from 0.3 μ to 1 μ (FIGURE 1).

From the characteristics of this agent as determined in chick embryos, it ecame clear that it was either a large virus, *Rickettsia*, or one of the PPLO. n addition, these groups of infectious agents were of prime suspicion since esions similar to those described above have been produced in pigs, sheep, oats, cows, and chickens by infectious agents belonging to the group of large irus or to PPLO.[1,3-11] A striking similarity between the electron micrographs f this agent and published electron micrographs of PPLO supported the sus-icion that we were dealing with one of the PPLO.[12] The morphologic resem-lance to PPLO, coupled with a failure to obtain growth on a medium[13] used successfully for growing PPLO from chickens, humans, cows, and goats, suggested that this polyserositis agent might be a more fastidious PPLO.

In order to pursue this thought, the basic PPLO medium (heart infusion broth or agar with 1 per cent Bacto-PPLO serum fraction) was modified by substituting other sera for the PPLO serum fraction. Chicken, horse, cow, pig, and rabbit sera, added at a concentration of 10 per cent, were tested for their growth-promoting capacity. Plates inoculated with chick embryo fluids containing the polyserositis agent were incubated aerobically, under increased CO_2 and decreased O_2 tension (candle jar), and anaerobically at 37° C. It was noticed on the plates supplemented with pig serum and incubated in a candle jar that extremely small colonies (approximately 0.01 mm. in diameter) seemed to have developed. Because of their size it was difficult to determine whether they were debris or colonies similar to the T (tiny) form of PPLO colonies isolated by Shepard[14] from cases of nongonococcal urethritis in humans. Shortly thereafter, in another experiment, one of the plates became contaminated with a colony of micrococcus and, around this colony, a satellite phenomenon was observed. Large, typical PPLO colonies (approximately 0.1 mm. in diameter) were found near the micrococcus, and the colonies of PPLO became smaller and smaller, finally resembling the T form, as the distance from the bacterial colony increased. Thus it was felt, from a consideration of the *in vivo* and *in vitro* characteristics of the polyserositis agent, that it was a PPLO.

Comparison of the chicken tendovaginitis agent with the pig polyserositis PPLO. It was wondered whether a similar satellite phenomenon might be demonstrated for the chicken tendovaginitis agent, since the characteristics of this agent, as determined in chick embryos[1] and by pathological manifestations,[15] although commensurate with the characteristics of large viruses, were also in harmony with the characteristics of PPLO. However, again, attempts by different laboratories to grow this agent as PPLO met with failure.[1,9-11] Five strains of the chicken tendovaginitis agent whose *in vivo* characteristics had been described,[1] were inoculated near a colony of *Micrococcus* on heart infusion agar with 10 per cent pig serum and incubated at 37° C. in a candle jar. Typical colonies of PPLO grew near the bacterial colony. The satellite phenomenon was demonstrated when the medium was supplemented with pig serum, turkey serum, chicken serum, cow serum, rabbit serum, but not horse serum or Bacto-PPLO serum fraction. A fresh 30 per cent boiled-water extract of yeast could be substituted for the growth-promoting activity present in the *Micrococcus* colony. Bacto-yeast extract, desoxyribonucleic acid, ribonucleic acid, and hog gastric mucin did not exhibit this activity. Heart infusion media containing 10 per cent fresh yeast extract and 10 per cent pig serum supported the growth of both the tendovaginitis and the polyserositis PPLO. Pig serum was superior to either turkey or chicken serum. It was possible to reisolate directly on this medium PPLO from the same spleens that had been the original source of the chick embryo–isolated polyserositis PPLO. These spleens had been frozen since the time of the original isolation. It has been noted that growth of certain strains of PPLO in media enriched with horse serum varied with the lot of the serum. Growth was enhanced by the addition of yeast extract.[16] Other investigators have also incorporated yeast extract in their PPLO media.[4,5] Obviously, however, not all strains of PPLO isolated from uch sources as chickens, pigs, and humans require the addition of fresh yeast xtract to the medium for growth.

Reproduction of Polyserositis

Pigs used for experimental infectious studies were of two types: colostrum-ree, and natural, sow-raised. The colostrum-free pigs were obtained either by hysterectomy approximately 3 days before term, or by catching pigs at birth in sterilized shrouds, before they came in contact with the floor. They were then placed in individual cages in an isolation unit (12 pigs per unit). For the first 4 weeks, they were fed cow's milk fortified with minerals, and then were gradually weaned to a commercial pig ration. At this time, the cages were removed from the isolation unit. It was hoped that this farrowing and isolation procedure would make it possible to obtain not only colostrum-free pigs, but also pigs essentially pathogen-free. Sow-raised pigs were allowed to nurse the sow under routine farm conditions. These pigs were weaned when approximately 6 weeks of age. At approximately 8 weeks of age they were placed in isolation units.

It was observed, when colostrum-free piglets (2 to 4 months of age), in isolation since birth, were inoculated intraperitoneally with the pig polyserositis PPLO that, in comparison with naturally developing cases on the pig farm, the gross symptoms of disease were quite mild: slight rise in temperature (105° F. approximately 3 to 4 days after inoculation, followed by a return to normal) with perhaps some sluggishness for the first week after inoculation. However, f these animals were necropsied 2 to 3 weeks after inoculation, it was possible to recover the PPLO from the liver, spleen, and joints. Also, symptoms of fibrinous pericarditis, pleuritis, and peritonitis were observed. Pigs not sacrificed appeared normal and gained weight properly; however, when slaughtered 3 to 4 months later, they too had scar tissue indicative of healed polyserositis. Obviously, even though we were reproducing the polyserositis lesions with PPLO, we were not reproducing the same disease as seen in the field, since the infected field pigs had high temperatures (107 to 108° F.) and died. The post-mortem examinations of these field pigs also revealed a hemorrhagic gastroenteritis and cystitis in addition to the polyserositis. Experiments were designed to reconcile this discrepancy between natural field cases and artificial cases in isolation.

The following experiments were performed (FIGURE 2) to determine whether the high fever, gastroenteritis, and death found in the field cases were due to stress involved in moving the pigs from warm, clean, tile, isolation units to the more rigorous conditions of outdoor life and competition in a hog lot, or whether the symptoms were more directly due to exposure to a fever-provoking, lethal, infectious agent present, but in equilibrium with sow-raised pigs.

Four groups of pigs (3 groups of colostrum-free animals and 1 sow-raised group) in 4 separate isolation units were infected when about 2½ months old with polyserositis PPLO. Post-mortem examination, plus reisolation of the PPLO of one of the colostrum-free pigs 2 weeks after injection with the PPLO, indicated that the infection had taken. Three months after infecting these 4 groups of pigs with PPLO, 1 group of colostrum-free pigs and 1 group of sow-raised pigs were moved from isolation to the company of pigs on the hog farm;

another group of colostrum-free pigs was moved to a "clean" hog lot (known not to have contained pigs for about 1 year); and the last group of colostrum-free pigs remained in the isolation unit. Four days later, the colostrum-free pigs placed on the hog farm began to die; they revealed, on post-mortem examination, polyserositis lesions of long standing: scars in the peritoneum, pleural cavity, and pericardium, and fresh-looking, hemorrhagic gastroenteritis, cystitis, and arthritis. The sow-raised pigs (in isolation for 3 months and returned to the farm) exhibited no gross signs of disease. Similarly treated colostrum-free pigs placed in the "clean" hog lot and those left in the isolation unit remained asymptomatic in the gross. Spleens and livers from colostrum-free pigs dying at the hog farm were negative for bacteria on culturing and staining. However, emulsions of these same spleens and livers, injected into the colostrum-free, polyserositis-infected litter mates remaining in isolation, produced high fever, gastroenteritis, cystitis, and death within 20 to 96 hours.

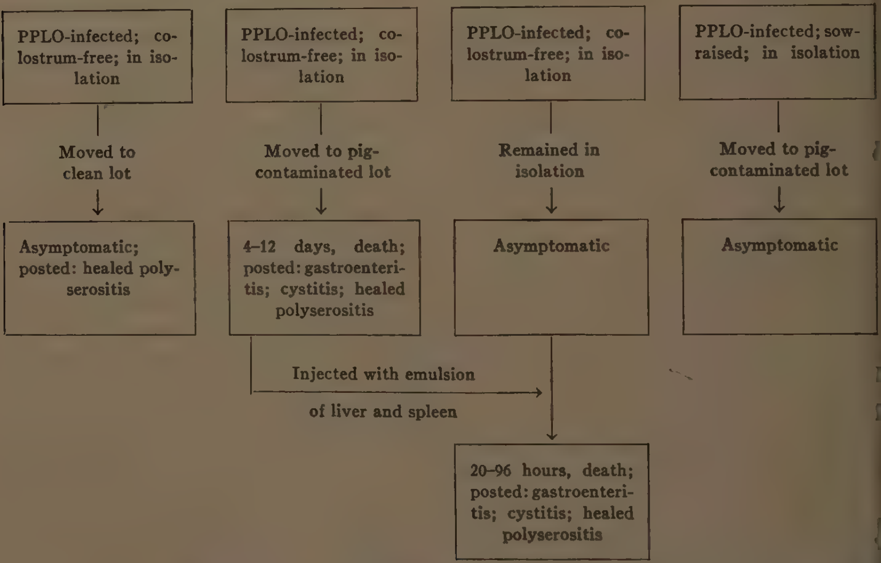
PPLO-infected; colostrum-free; in isolation
PPLO-infected; colostrum-free; in isolation
PPLO-infected; colostrum-free; in isolation
PPLO-infected; sow-raised; in isolation
Moved to clean lot
Moved to pig-contaminated lot
Remained in isolation
Moved to pig-contaminated lot
Asymptomatic; posted: healed polyserositis
4–12 days, death; posted: gastroenteritis; cystitis; healed polyserositis
Asymptomatic
Asymptomatic
Injected with emulsion
of liver and spleen
20–96 hours, death; posted: gastroenteritis; cystitis; healed polyserositis

FIGURE 2. Reproduction of pig polyserositis as seen in the field. Pigs, in isolation, were infected with PPLO and, three months later, were moved to various locations.

From this it was concluded that another infectious agent was responsible for the high fever, gastroenteritis, and death seen in the field cases. Penicillin, streptomycin, and Terramycin protected infected pigs; therefore, a bacterium was suspected and eventually isolated from infectious spleen and liver on a blood plate near a colony of *Micrococcus*. This bacterium was identified as *Hemophilus influenzae suis*.[17] Workers in Sweden and Germany have attempted to identify *Hemophilus* in field cases in naturally raised pigs.[18,19]

Two factors may account for difficulties in isolating *Hemophilus* in this syndrome:

(1) This particular strain had strict requirements for the *X* and *V* factor. No discernible growth was visible on blood plates without *Micrococcus*; extremely scant growth occurred on a chocolate-blood plate; and growth was

best near a *Micrococcus* colony on a chocolate-blood plate supplemented with Bacto-supplement B. Some of the strains isolated subsequently were not as difficult to grow.

(2) If isolations were attempted from liver and spleen at the time of death or after the animals had been sick a few days, one found very few if any *Hemophilus* remaining in the visceral organs. It was noticed, however, that even though few remained in the visceral organs at this time, it was easy to isolate large numbers from the brain simply by inserting a cotton swab into the brain through a hole chiseled in the cranium. Most of the experimental animals died in 24 to 72 hours; however, a few that lived for 6 to 14 days were negative when the visceral organs were cultured, but good growth of *Hemophilus* was obtained from the brain. Thus, it seemed that, the longer the animal lived after first symptoms, the harder it was to isolate from the liver and spleen, while little difficulty was encountered in making isolations from the brain.

This *Hemophilus*, passed on blood plates 3 times and then grown in chick embryos (killed 9-day-old embryos in 24 to 48 hours) reproduced in colostrum-free pigs the hemorrhagic gastroenteritis, cystitis, and arthritis, as well as severe congestion in the liver and kidneys and edema in the lungs. In addition, excess fluid often was found in the peritoneal and pleural cavity. *Hemophilus* have been reisolated from blood, liver, spleen, kidneys, lungs, brain, and joints. As few as 200 *Hemophilus* injected intramuscularly into colostrum-free pigs can kill in 36 to 96 hours. However, more than 10^6 *Hemophilus* injected in sow-raised litter mates have not caused any visible symptoms. Large numbers of organisms injected in colostrum-free pigs produce, within 3 hours, high fever and limping in the injected leg. These symptoms abate approximately 6 hours after injection, only to reappear the next day and lead rapidly (24 to 36 hours) to death. In this respect the initial symptoms seem to be the result of a toxic reaction.

Attempts to show that a combination of PPLO and *Hemophilus* was more pathogenic than either alone have been negative. This, in part, may be explained by the fact that our experimental colostrum-free animals were so susceptible to *Hemophilus* that it was not possible to obtain gradations in susceptibility. However, it has been possible to isolate PPLO, but not *Hemophilus*, from the spleen of doubly infected pigs; from the brain, *Hemophilus* was isolated, but not PPLO. It is felt the exact ecological relationship of these 2 microorganisms in field cases needs further amplification; for example, only recently a nonbacterial agent cytopathogenic for pig kidney tissue cultures has been isolated from the intestines of a field case.[20] It is noteworthy that, in a type of fowl coryza, both PPLO and *H. gallinarum* are needed in order to reproduce the lesions seen in the disease.[21] It was felt, in this connection, that in chronic respiratory disease of chickens a combination of PPLO and bacteria produced the severe caseous lesions seen in field cases.[13]

Summary

It has been demonstrated that at least two different microorganisms are responsible for the lesions seen in the field cases of polyserositis with arthritis. One, a fastidious pleuropneumonialike organism (requiring a medium supplemented with fresh yeast extract and pig serum for growth), had the capacity to inflame serosal and synovial membranes, leading to peritonitis, pleuritis, pericarditis, and arthritis. The other agent, *H. influenzae suis* produced high fever, anorexia, unsteady gait, weakness in hindquarters, hemorrhagic gastroenteritis, cystitis, and death.

It is felt, in retrospect, that similar fastidious PPLO are the etiological agents of tendovaginitis with arthritis in chickens.

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CHARACTERISTICS OF PPLO ISOLATED FROM THE GENITAL AND RESPIRATORY TRACTS OF CATTLE*

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Pleuropneumonialike organisms (PPLO) were isolated from the genital tracts of cattle by D. G. ff. Edward and by B. E. Albertsen. The study described in this report was undertaken to determine if such organisms were common in West Virginia and if they were similar to previously described PPLO. One of the PPLO was isolated from the respiratory tracts of cattle showing signs of shipping fever.[1] Therefore, it was desirable to learn whether the organisms were common in normal-appearing animals and whether these organisms were similar to those isolated from the genital tract.

Review of Literature

The literature concerning PPLO isolated from the genital tract has been reviewed.[2] Strains have been described from cattle (*Mycoplasma bovigenitalium* and P or pathogenic strains) and from sewage and other sources (*M. Laidlawii* and S or saprophytic strains).[3] The P strains require horse serum, ascitic fluid, or other special enrichment in the media for growth. The S strains are capable of growing in media without special enrichment. The P strains were isolated from cattle with records of poor breeding efficiency.[4] In heifers inoculated *in utero*, conception was not prevented, but inflammation of the uterus resulted.[5] Albertsen isolated the P strain from 25 of 26 bulls examined. He concluded that these organisms were not a cause of infertility in cattle.[6]

A high proportion of the cultures from throat and vaginal samples taken from dogs revealed PPLO.[7] *M. hyorhinis* was recovered from the nasal passages of swine by Carter and McKay,[8] and by Switzer.[9] The latter felt that the nasal cavity was a natural habitat of swine PPLO.

Material and Methods

The base medium used for cultivating PPLO consisted of dehydrated beef heart infusion with PPLO broth or agar (Difco). To this was added 10 to 20 per cent horse serum, 10 per cent yeast hydrolyzate, 1000 U. of penicillin per milliliter, and thallium acetate to make a 1:4000 solution. Neither thallium acetate nor penicillin was added to the agar. The *p*H was adjusted to 7.8.

The vagina of the cows was cultured by placing a cotton tampon in the anterior vagina. This was removed in 30 min. An alternate method was to wash the anterior vagina with a Pierce pipette[10] using PPLO broth. A Bartlett tube[11] was used to obtain mucus from the glans penis. Mucus was obtained from the sheath and nose with cotton swabs. Semen was collected with an artificial vagina. With either method the collected material was placed in PPLO broth, transferred after 24 hours, and then every 7 days. Nine passages were made before the material was discarded as negative or contaminated. On each broth transfer 0.05 ml. was placed on PPLO agar to be observed for growth and colony characteristics.

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For fermentation studies, the base medium, yeast hydrolyzate and horse serum, were fermented with *Aerobacter aerogenes* for 18 hours and filtered. This filtrate plus 0.5 per cent of the desired carbohydrate was sterilized by Seitz filtration. Phenol red (0.0025 per cent) was used as an indicator. Acid reaction was determined by color change at the end of 7 days. Each carbohydrate was plated on hard agar to check for growth or contamination.

Five per cent defibrinated horse, rabbit, or sheep blood was added to the basal PPLO agar to check for hemolysis.

Selected isolates were placed on McConkey agar, McConkey broth, Brewer's thioglycollate, thiol, MR-VP, Sim, and tryptose phosphate broth.

Each culture was plated on hard agar and examined for growth at room temperature (22° C.). Growth on hard agar without the addition of horse serum or yeast hydrolyzate was determined.

Growth inhibitory tests using hyperimmunized rabbit serum were run in a manner similar to that employed by Edward and Fitzgerald.[12] The hyperimmunized rabbit serum was prepared by intravenous inoculation of rabbits with 1 ml. of PPLO culture. Inoculations were given 3, 6, 10, 12, and 16 days after the initial one. The average number of PPLO per milliliter of inoculant ranged from 5.47×10^7 to 8.54×10^{10}. The rabbits were bled on the seventeenth day. The serum was inactivated by incubation in a water bath at 55° C. for 30 min. and stored at 4° C. until used. The following strains of PPLO were used: A-5969* from the trachea, AC-1791 from joint fluid of chickens, AC-643 from mucus of a calf with shipping fever, A-86-N and H-88-N from the nose of cows, H-18-S from the sheath of a bull, and G-14-GP and H-4-GP from the glans penis.

Colony characteristics were classified on the third day of incubation as of four types: (1) smooth edges with center spots, (2) smooth edges without center spots, (3) rough edges with center spots, and (4) rough edges without centers.

Microscopic examination of colonies was made by fixing the colony to a glass slide by the use of Bouin's solution. The colonies were examined under a phase-contrast microscope or were stained with Giemsa and examined under an ordinary microscope. Other colonies were stained with Dienes' stain by making a thin film of the stain on a coverslip. An agar block containing colonies was cut out and the stained cover slip was placed on the block. These were observed after 30 min. to determine the amount of stain taken up by the colony.

One strain, G-14-GP, isolated from the glans penis of a bull, was infused into the uterus of a cow. Strain AC-643, isolated from a nasal discharge of a calf with shipping fever, was inoculated intravenously and intranasally into a 3-day-old calf.

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* Obtained from E. L. Jungherr, University of Connecticut, Storrs, Conn.

Results

The number of samples and the number of isolations of PPLO from the cows and bulls are recorded in TABLE 1. No isolations were obtained from the anterior vagina in these trials.

TABLE 1
ISOLATION OF PPLO FROM THE ANTERIOR VAGINA, SHEATH, SEMEN, GLANS PENIS, AND NOSE OF CATTLE

Source	No. of isolations	No. of animals	No. of samples	No. contaminated
Anterior vagina	0	93	133	9
Semen	0	30	60	4
Sheath	15	30	60	11
Glans penis	19	30	60	7
Nose of cows	11	30	30	7
Nose of bulls	3	10	10	3

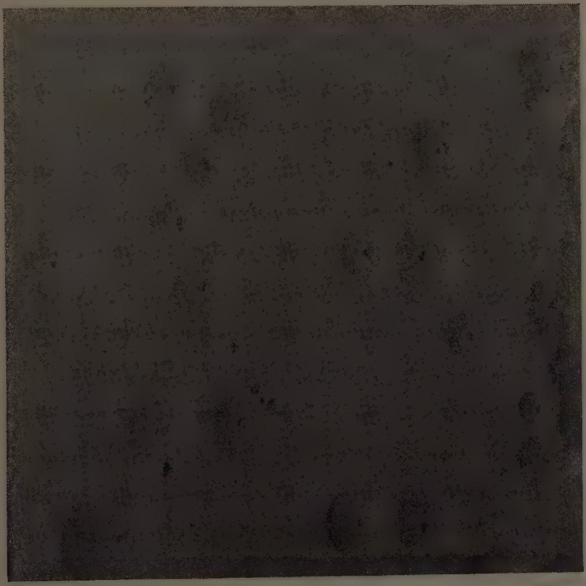

FIGURE 1. Type-1 growth, showing smooth edges with centers. From sheath of bull (502); growth 7 days old, fifth passage. ×60.

Illustrations of the various types of colonies observed are shown in FIGURES 1 to 4. In the first three passages, most of the cultures showed rough growth with and without centers, but when individual colonies were subcultured the growth ranged from smooth to rough. In later passages the colonies were usually smooth (growth types 1 and 2) in 24- to 72-hour cultures. In 3- to 5-day cultures, Type-1 growth was usually observed. In older cultures, all 4 types of growth were observed. When rough colonies were subcultured, Types 1 to 4 were observed.

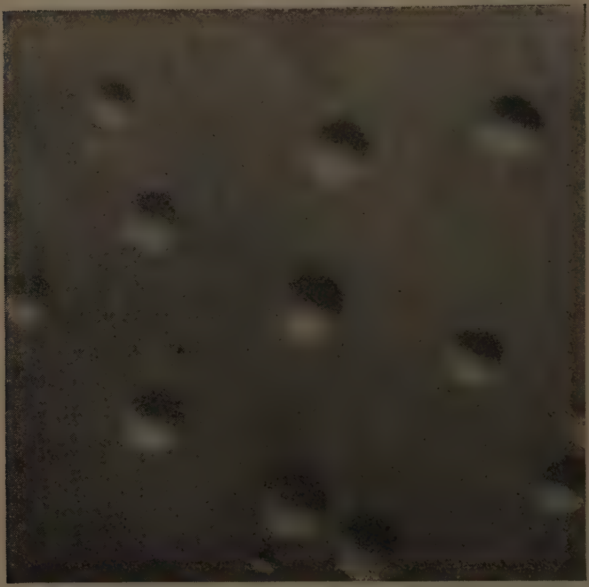

Figure 2. Type-2 growth, showing smooth edges without centers. From nose of cow (G-83); growth 48 hours old, seventh passage. ×60.

Figure 3. Type-3 growth, showing rough edges with centers. From sheath of bu (H-20); growth 12 days old, eighth passage. ×60.

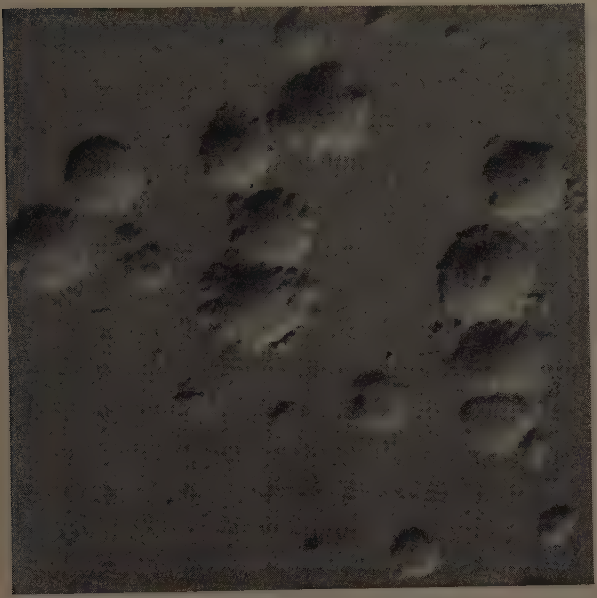

FIGURE 4. Type-4 growth, showing rough edges without centers. A few colonies at the edges have centers. From glans penis of bull (A-8); growth 48 hours old, second passage. ×60.

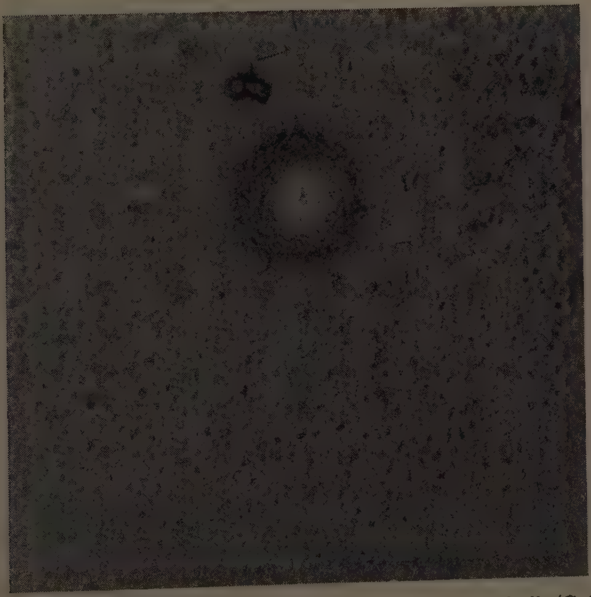

FIGURE 5. Giemsa-stained PPLO colony from glans penis of bull (G 14); growth 48 hours old, fourth passage. ×480.

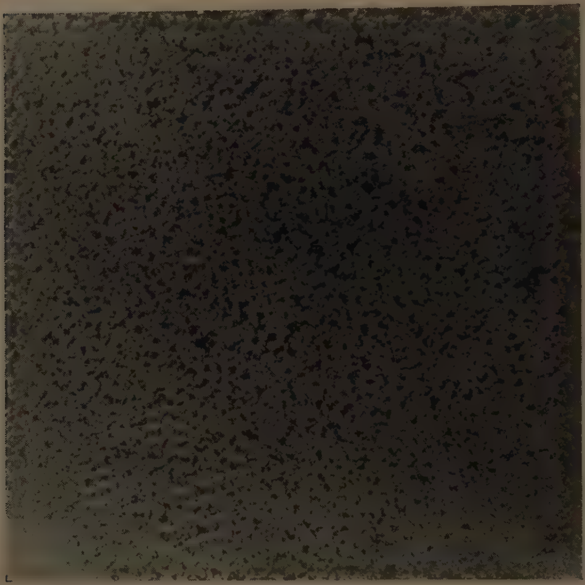

FIGURE 6. Giemsa-stained PPLO colony from nose of cow (H-86); growth 48 hours old, third passage. ×480.

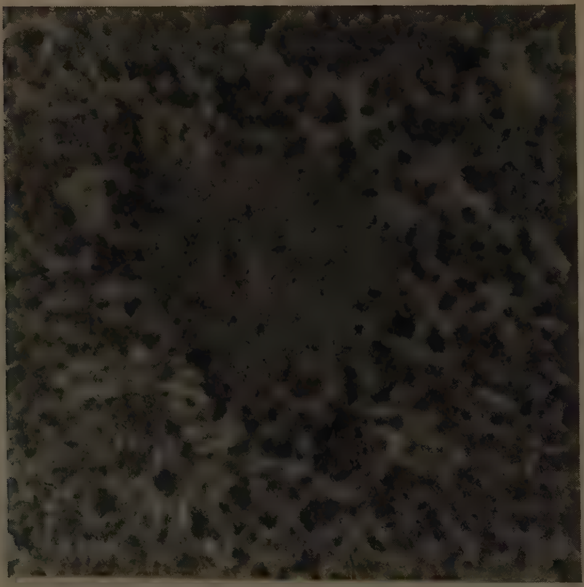

FIGURE 7. Giemsa-stained PPLO colony from sheath of bull (H-20-S); growth 48 hours old. ×1200.

Colonies that had been stained on hard agar showed a dark center surrounded by a peripheral zone of finely granulated particles (FIGURE 5). Those that did not have centers were made up of fine granular bodies (FIGURE 6). Higher magnification revealed coccoid bodies and indistinguishable masses (FIGURE 7). Examination of fluid cultures frequently revealed masses containing coccoid forms and polymorphic-type structure. An occasional mobile form was seen.

TABLE 2

TYPE OF COLONY, GROWTH AT ROOM TEMPERATURE, AND FERMENTATION REACTION OF PPLO ISOLATED FROM THE NOSE, GLANS PENIS, AND SHEATH OF CATTLE, AND OF TWO ISOLATES FROM CHICKENS

PPLO isolation*	Growth at 22° C.†	Type of colony	Fermentation‡							
			Glucose	Fructose	Galactose	Maltose	Mannose	Dextrin	Starch	Glycogen
Avian										
A-5969-T	−	1, 2, 4	+	+	(+)	+	+	+	+	+
AC-1791-H	−	1, 2, 4	+	+	(+)	+	+	+	+	+
Cattle										
AC-643-N	−	1, 2	−	−	−	−	−	−	−	−
H-86-N	+	1, 2	+	+	+	+	−	+	+	+
J-16-N	−	1	+	−	−	+	−	+	+	+
H-88-N	+	1, 3	−	−	−	−	−	(+)	+	(+)
J-663-N	(+)	1, 4	(+)	(+)	(+)	−	−	−	(+)	(+)
J-19-S	+	2	(±)	−	−	−	−	+	+	+
J-18-S	+	1, 2, 3	(+)	−	−	(+)	−	+	+	+
J-18-S	+	1, 2	−	−	−	−	−	+	+	+
502-S	+	1, 2	(±)	−	−	−	−	+	+	+
J-16-S	+	1, 4	(+)	−	−	−	−	+	(+)	(+)
H-20-S	+	1, 3	(±)	−	−	−	−	(±)	+	+
A-8-S	(+)	2, 1	−	−	−	+	−	+	+	+
J-22-S	+	1, 2	−	−	−	−	−	+	+	+
J-20-GP	+	1	(+)	−	−	+	−	+	+	+
J-20-GP	+	1	+	+	−	+	−	+	+	+
H-4-GP	+	1, 2	(±)	−	−	(±)	−	−	+	+
H-18-GP	+	2, 4	(±)	−	−	+	−	+	(±)	+
G-15-GP	(+)	2, 3	(±)	−	−	−	−	(±)	(+)	+
A-8-GP	(+)	1, 2	−	−	−	+	−	+	+	+
G-14-GP	+	1, 2	−	−	−	+	−	+	+	+
J-21-GP	+	2	−	−	−	+	−	+	+	+

* Final letters indicate: T, trachea; H, hock; N, nose; S, sheath; and GP, glans penis.

† Symbols represent: −, no growth; +, growth; and (+), slight growth.

‡ Symbols represent: −, no change; +, complete change; (+), partial change; and (±), slight change.

All cultures except AC-643 and 16-N from cattle grew at room temperature, and all except AC-643 grew on serum-free agar and broth. Generally, Type 1 and Type 2 growth was observed (TABLE 2). The growth in fluid medium was clear except in the case of AC-643, which was cloudy. The centrifugate was white. Horse, rabbit, or chicken blood were not hemolyzed. No chicken strains grew at room temperature or in serum-free broth, except strain 1791, which grew on serum-free agar.

The fermentation of carbohydrates was difficult to evaluate (TABLE 2). None of the strains tested fermented mannose. Generally all strains fermented dextrin, soluble potato starch, and glycogen; however, two strains from the nose did not ferment dextrin, and gave variable reactions on the sugars tested. The strains from the sheath did not ferment fructose, galactose, or mannose, and only two strains fermented maltose. The glucose reaction was variable. With one exception, the strains from the glans penis did not ferment fructose or galactose. Maltose was generally fermented. Culture AC-643 did not ferment any of the carbohydrates tested, and the poultry strains fermented all carbohydrates tested.

All strains were carried for from three to six passages in inhibitor-free broth. No bacteria were demonstrable in these cultures. Occasional bacteria were found on agar plates, but these were not in the zone of inoculation.

Strains from cattle (except AC-643) were carried for from three to six passages in Brewer's thioglycollate media, after which no further growth could be demonstrated when they were plated on hard agar. No growth occurred on McConkey agar and broth, thiol, MR-VP, Sim, or tryptose phosphate broth.

The inhibitory tests of cattle PPLO with hyperimmunized rabbit serum were negative, with the exception of 86-N, which showed slight inhibition in the homologous serum at a concentration of 1:10 to 1:40, but not against sera from other strains. The 2 chicken strains were inhibited by homologous and heterologous sera of rabbits inoculated with the 2 strains of PPLO isolated from chickens, but were not inhibited by sera from the cattle strains.

Approximately 300,000,000 organisms (strain G-14-GP from the glans penis) were infused into the uterus of one cow. Biopsies of the uterine mucosa were taken every 7 days after infusion. PPLO were isolated when the fourth, fifth, sixth, and seventh biopsies were taken.

At 28 days after inoculation, the surface epithelium was slightly disorganized and an accumulation of neutrophils and mucus was present in the lumen. There was no detachment from the subepithelial stroma. The glandular epithelium was moderately degenerated, with a small amount of cell debris in the lumen of a few glands. In later biopsies there was a more pronounced neutrophilic and lymphocytic infiltration in the epithelium and the superficial layer of the stroma. Eosinophils were present. These changes occurred up to the twelfth biopsy. The thirteenth and fourteenth biopsies revealed a gradual improvement.

Strain AC-643 was inoculated intranasally and intravenously into a 3-day-old calf. There were no signs of disease for 6 weeks. No PPLO were isolated before inoculation, but on the second and third weeks following inoculation PPLO were isolated from the mouth of the inoculated calf, but not from the control calf. No abnormal changes were noted, and no PPLO were isolated when the calf was autopsied 6 weeks after inoculation.

Discussion

The strains isolated from cattle did not have the characteristics of previously described PPLO obtained from cattle, with the exception of AC-643, for which enriched medium was not required for growth. The strains isolated grew at room temperature and did not hemolyze horse blood. In these respects they resembled the S strains described by Edward. However, in that they did not hemolyze horse blood and gave different sugar reactions, they differed from those described by Edward and Albertsen. The fact that the organisms were not inhibited by hyperimmunized rabbit serum suggests that they are L-phase bacteria. This is strongly suggested by the variable fermentations obtained with the various carbohydrates, and also by the growth of some strains in Brewer's thioglycollate medium; however, this could represent merely a dilution of the PPLO inoculated into the original tube. On the other hand, if the organisms isolated in this study can be shown to be pathogenic for the uterine mucosa, it makes little difference whether they are PPLO or L-phase bacteria. Additional work is needed to classify these organisms properly.

Summary

Organisms that resembled PPLO in colonial characteristics were isolated from the genital tracts and noses of cattle. However, biochemical reactions suggest that they are either L-phase bacteria or PPLO that have not been previously described.

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PATTERNS OF REACTION IN INFECTION WITH A VIRULENT FORM OF PPLO FROM GOATS*

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Earlier work[1,2] has demonstrated that the pleuropneumonialike organisms (PPLO) isolated from a septicemic and arthritic disease of goats are also pathogenic for sheep and swine by intravenous and intraperitoneal inoculation. Subsequent experiments have been undertaken to define the host range of this infection and to study the patterns of reaction in sheep exposed by various routes.

General Experimental Procedures

The K strain of the goat arthritis PPLO, as third-passage yolk-sac material frozen for 4 years at approximately −20° C., was inoculated into PPLO broth (Difco) supplemented with 1 per cent yeast hydrolyzate and 10 per cent horse serum. After 5 days' incubation, the medium was found to contain approximately 1 million viable organisms per milliliter. This culture constituted the inoculum for the first 2 experiments. The inoculum for the third experiment consisted of a similar broth culture seeded with joint exudate obtained from an animal used in the second experiment. The joint had been frozen 4 months at −20° C. Unlike the practice in earlier work, ground agar medium was not added to either of these inocula.

Methods for recovery of the organism from fluids or tissues were those used earlier with swine.[2] The agglutination test employed was one slightly modified from that of Priestley.[3] Agglutination tests of all preinoculation sera were negative.

First Experiment: Response in Clinically Unaffected Hosts

Experimental hosts, other than sheep, were as follows: an aged Arabian mare weighing 900-lb.; 2 calves (a 400-lb. crossbred female and a 200-lb. Jersey male); 4 female beagles aged 13 to 15 months and weighing 24 to 27 lb.; 4 rabbits (2 females of 5 kg. each and 2 males of 3 kg.; 6 male Sprague-Dawley rats averaging 320 gm.; 6 male guinea pigs averaging 230 gm.; 6 male Webster Swiss mice averaging 22 gm.; 6 male broad-breasted bronze turkeys of about 4 kg. each; and 5 single-comb White Leghorn hens of about 2 kg. each. Doses and routes of inoculation are given in TABLE 1. These animals were sacrificed for examination on the fifteenth day after exposure, except for the dogs and calves, which were observed for a longer period and not autopsied.

None of these hosts showed any clinical evidence of infection. Temperatures remained normal and behavior was unaffected. PPLO were not recovered from any of the specimens. An agglutination reaction with undiluted serum was found in calves, turkeys, chickens, and one rabbit (TABLE 1). Several of the birds gave positive reactions with serum dilutions of 1:10.

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* The work reported in this article was supported in part by a grant from Abbott Laboratories, North Chicago, Ill.

Second Experiment: Sheep Exposed by Various Routes

Twenty-two sheep from 3 sources were used in this work. Except for 5 yearlings, these were lambs weighing 50 to 70 lb. All received 1 ml. of inoculum, with 0.5 ml. given by the intradermal route. Routes of exposure are given in TABLE 2. Intratracheal injections were made with a hypodermic needle through the skin and other tissues in the middle of the neck. Oral inoculation consisted of spraying the inoculum into the posterior oral cavity and pharynx with a syringe.

TABLE 1

RESPONSE IN CLINICALLY UNAFFECTED HOSTS

Host	Dose and route	PPLO recovered*	Agglutination†	
			Undiluted	1:10
Horses	1 ml. I.V.	0	0/1	0/1
Calves	1 ml. I.V.	0	2/2	0/2
Dogs	1 ml. I.V.	0	0/4	0/4
Rabbits	0.5 ml. I.V.	0	1/4	0/4
Rats	0.5 ml. I.P.	0	0/6	0/6
Guinea pigs	0.5 ml. I.P.	0	0/6	0/6
Mice	0.5 ml. I.P.	0	0/6	0/6
Turkeys	0.5 ml. each I.V./A.S.‡	0	6/6	4/6
Chickens	0.5 ml. each I.V./A.S.‡	0	5/5	2/5

* Spleen and blood cultured; blood only in calves and dogs.

† Sera drawn on fifteenth day. Numerator gives number positive; denominator, number exposed and tested.

‡ A.S. represents injection into abdominal air sac.

TABLE 2

EXPOSURE OF SHEEP BY VARIOUS ROUTES*

Route†	Day of death	Arthritis	Serositis	Meningitis	PPLO recovered	Agglutination
S.C.	2	0	0	0	+	
I.C.	3	0	0	+	+	
I.M.	3	0	0	0	+	
I.T.	4	0	0	0	+	
I.T.	4	0	0	0	+	0
I.V.	5	+	+	0	+	±
I.D.	6	+	+	+	+	0
S.C.	6	+	+	+	+	+
I.D.	6	+	+	+	+	+
I.C.	6	+	+	+	+	+
I.T.	7	+	0	0	+	+
I.V.	7	+	+	+	+	+
I.T.	8	+	+	0	+	0
Oral	11	0	+	+	+	+
I.M.	15	0	0		0	0
3 oral and 4 contact controls					0	0

* Individual lambs were given 1 ml., or 0.5 ml. intradermally.

† S.C. represents subcutaneous introduction of the organism; I.C., intracisternal; I.T., intratracheal; and I.D., intradermal.

With the exception of 1 animal to be described separately, the parenterally inoculated sheep may be reported as a group regardless of the route of exposure (TABLE 2). After an incubation period of 1 to 3 days the animals showed hyperthermia (usually to 106 to 108° F.), anorexia, marked depression and, except for 3 that died very early, varying degrees of lameness. The course of the disease ranged from 2 to 6 days. PPLO were isolated from all specimens examined. The specimens comprised blood, spleen and usually joint exudate, lung serous cavity fluid, regional lymph node, and tissue from the inoculation site. One animal showed a suspicious agglutination reaction at 5 days, 5 were positive at 6 or 7 days, and 3 were negative at 4 to 8 days. The 4 cases dying earliest were not tested.

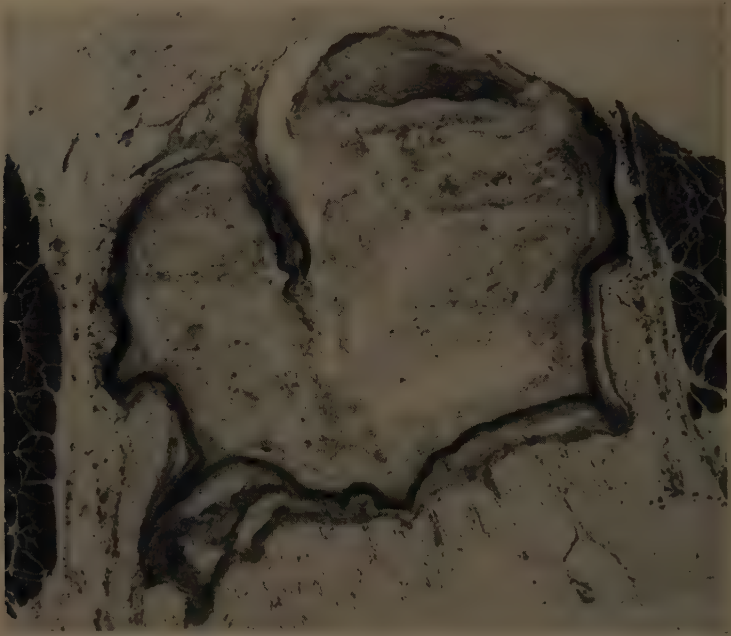

FIGURE 1. Transverse section showing atlanto-occipital synovitis after intradermal inoculation. Lumen is filled with fibrin.

The lesions, too, were of a pattern not specifically related to the route of infection. The 5 animals that died from the second through the fourth postinoculation days showed only serosal petechiae, mild splenic enlargement, a modest increase in serous cavity fluid, and acute serous lymphadenitis. Arthritis was not observed, and meningitis was evident only in the lamb inoculated intracisternally. Beginning on the fifth day, a fibrinopurulent synovitis, serositis, and meningitis (FIGURES 1 to 3) became well developed in most of the remaining animals (TABLE 2). These lesions appeared in one or more animals inoculated by each route, except that meningitis was absent in the intratracheally exposed group.

Leukocytic infiltration, edema, and necrosis occurred at the site of inoculation in many of these 13 animals (FIGURE 4). At most, this reaction occupied a volume of only a few cubic centimeters, except that in intravenously and intratracheally inoculated sheep a massive cellulitis extended from the point of injection to the thoracic inlet (FIGURES 5 and 6).

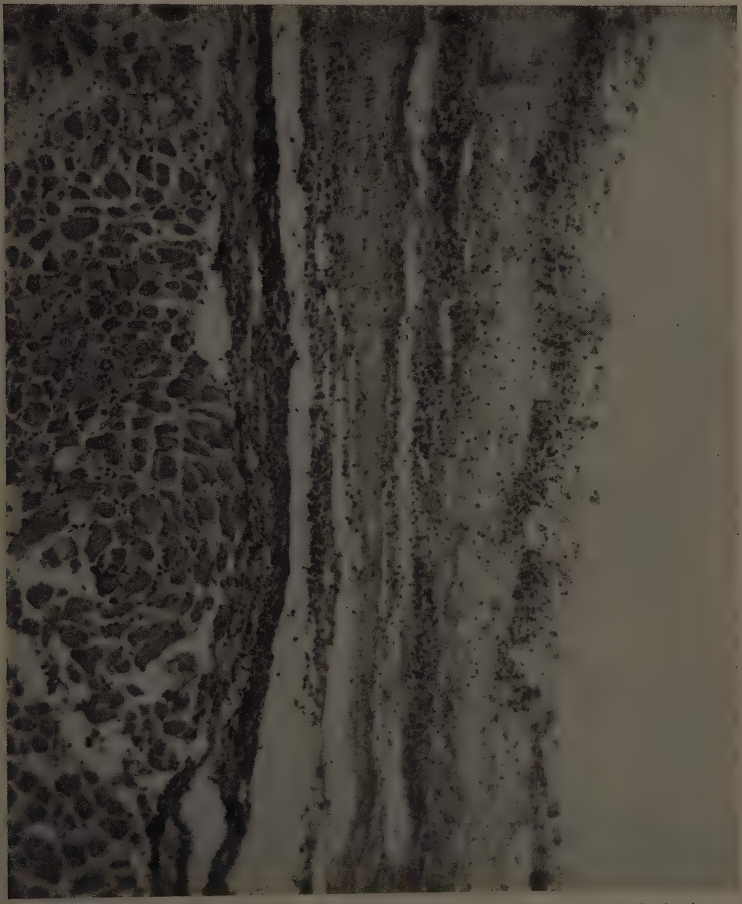

FIGURE 2. Peritonitis over the diaphragm (intratracheal inoculation).

The single sheep infected orally first showed symptoms on the sixth day and died on the eleventh. Lameness and other typical signs were observed. PPLO were isolated from blood and spleen, but not from a joint. The agglutination reaction was positive. Serositis and meningitis were well developed, but arthritis was absent except for murky fluid in the carpal joints.

Neither intratracheal nor other routes of exposure led to the appearance of frank pneumonia. Unfortunately, all lambs from one source, and only these, showed an intercurrent subacute bronchiolitis and atelectasis suggestive of that produced by intratracheal inoculation of ovine pneumonia virus. The latter agent produces a transient hyperthermia, some cough, and almost no deaths. The same picture was seen in another lamb of this source to which nothing had been done. Of the 4 lambs given PPLO intratracheally, two were not of this origin and showed no pulmonary lesions.

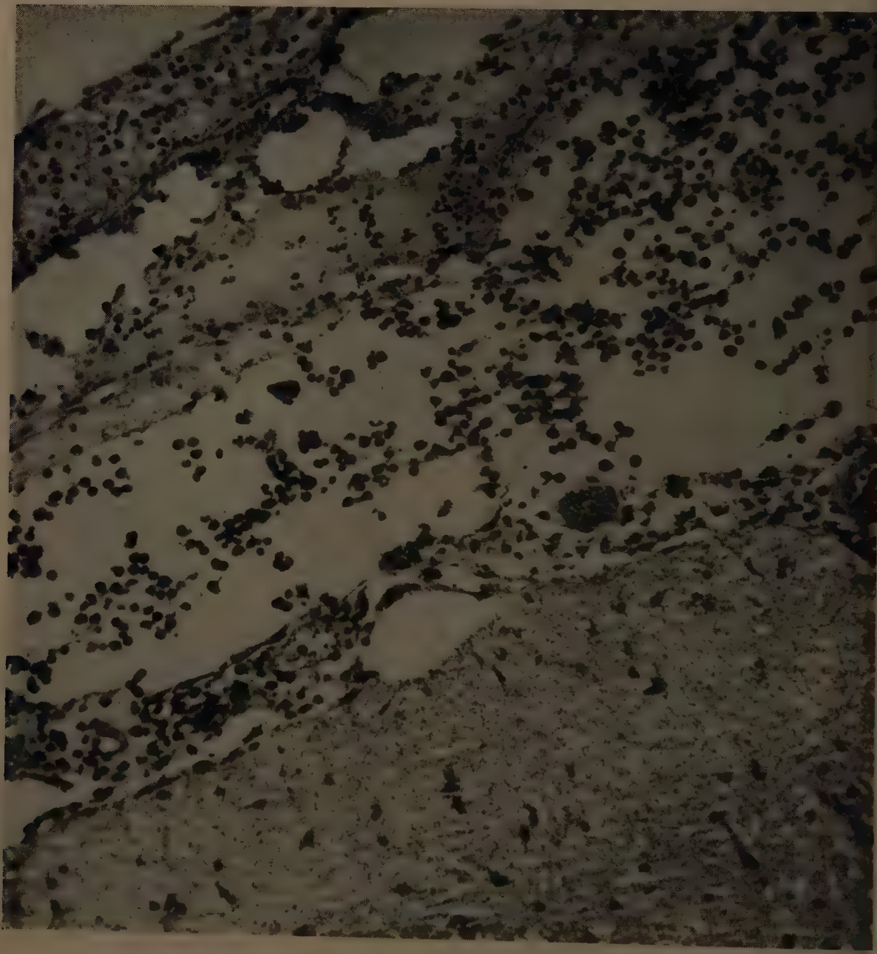

FIGURE 3. Leptomeningitis with fibrin (intradermal inoculation).

The exception to the usual pattern was a yearling ram inoculated intramuscularly. This animal showed a temperature of 105° F. on the fourth day and was distinctly lame from the third through the sixth days. For the next few days he hobbled on this leg only when actively exercised. He appeared to be well when sacrificed on the fifteenth day. PPLO could not be recovered from blood, spleen, joint, popliteal lymph node, or peritoneal fluid. The agglutination reaction was negative. There was an excess of clear peritoneal luid, and the involved popliteal lymph node was inflamed. In the nearby site of inoculation was a 1- × 4-cm. mass of blood, fibrin, and disintegrating neutrophils surrounded by a thick capsule of granulation tissue. Unfortunately, this lesion was not examined bacteriologically.

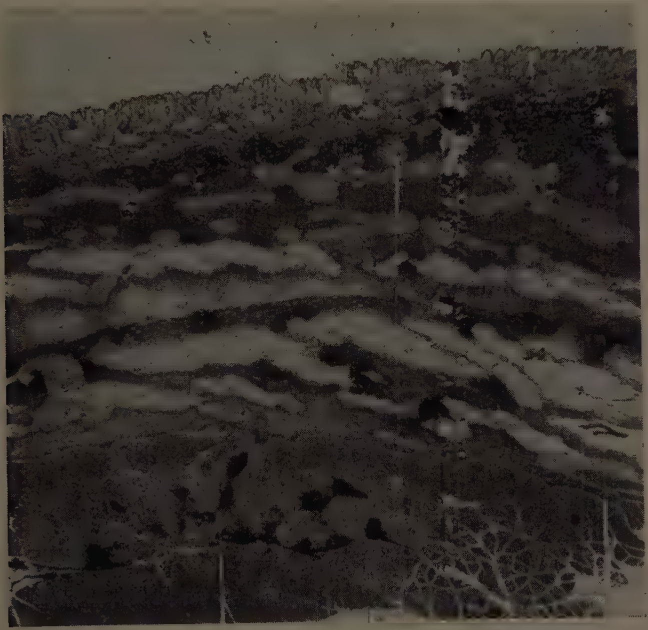

Figure 4. Local lesion in dermis, subcutis, and muscle at site of intradermal inoculation

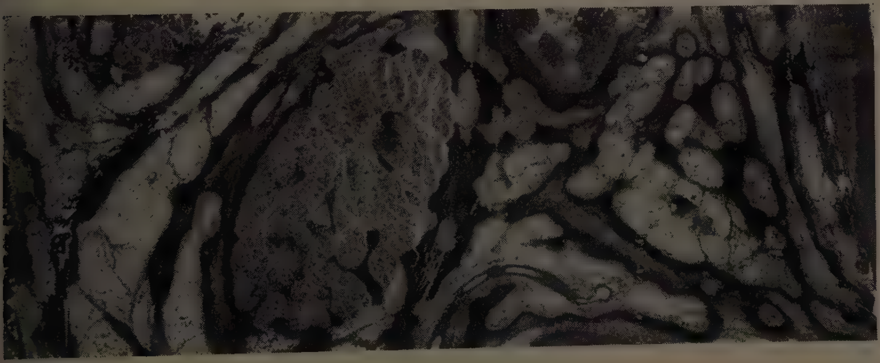

Figure 5. Cellulitis in neck over trachea (*right*) in area of intravenous inoculation.

All of the unaffected survivors (3 orally infected and 4 by contact) showed negative agglutination reactions. One of each group was sacrificed and no lesions were found nor were PPLO recovered from visceral specimens. The remaining animals were observed clinically until the twenty-third day.

Third Experiment: Sheep Exposed by Additional Routes

In this experiment 3 lambs were exposed for 10 min. to an aerosol of 20 ml of broth culture sprayed intermittently into a plastic hood with a poultr vaccine sprayer; in 3 others the skin of the side was scarified and cultur

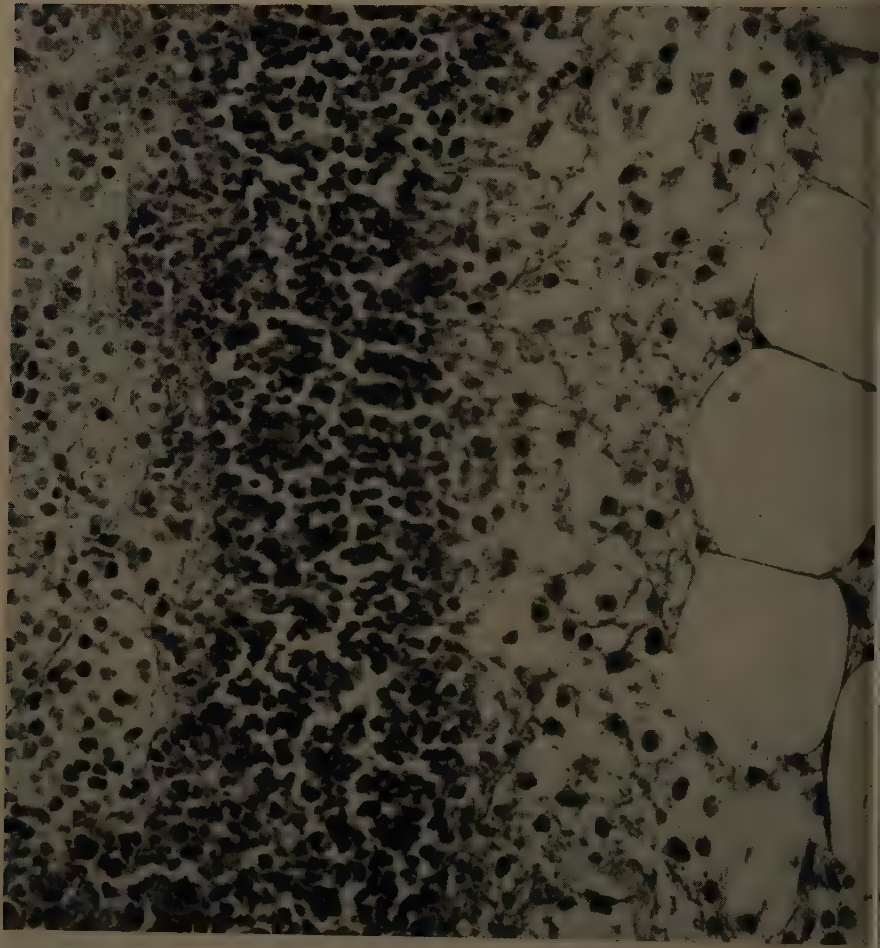

FIGURE 6. Higher magnification of area shown in FIGURE 5. Necrosis (*left*), neutrophilic infiltration (*center*), and macrophages in adipose tissue (*right*).

rubbed in according to the method for infecting swine with the erysipelas organism.[4] At the same time, as controls and to determine the influence of trauma in localization, 3 sheep were inoculated intradermally with 1 ml. of broth culture into the left elbow fold and crushing trauma was applied to the right fold without breaking the skin. On the second day after inoculation, these 3 animals were bled from the jugular vein so as to ensure an escape of blood into the perivenous connective tissue.

One of the lambs exposed to the aerosol was affected typically and, when sacrificed on the ninth day, showed arthritis, serositis, and meningitis. PPLO were recovered from blood, spleen, joints, and mediastinal lymph node. The agglutination reaction was positive.

The other 2 animals exposed to the aerosol and the 3 lambs exposed by scarification remained unaffected. On the twenty-third day after initial exposure these survivors showed no agglutinins and were challenged by intradermal inoculation of 1 ml. of broth culture grown from a lyophilized aliquot of that used for seeding the original inoculum. None was affected, except for one animal previously exposed by skin scarification. This lamb was sacrificed on the twelfth day and showed severe arthritis and some pericarditis. The agglutination reaction was negative, but PPLO were recovered in large numbers from a joint, and a few were isolated from the spleen. All other lambs were still negative to the agglutination reaction.

TABLE 3

EXPOSURE OF SHEEP BY OTHER ROUTES

Initial exposure*	Day of death	Lesions	PPLO recovered	Agglutination	I.D. re-exposure at 23 days			
					Day of death	Lesions	PPLO recovered	Agglutination
Aerosol	9	+	+	+				
Aerosol	Unaffected			0	Unaffected			0
Aerosol	Unaffected			0	Unaffected			0
Scarified	Unaffected			0	Unaffected			0
Scarified	Unaffected			0	Unaffected			0
Scarified	Unaffected			0	12	+	+	0
1 ml. I.D.	6	+						
1 ml. I.D.	6	+						
1 ml. I.D.	6	+						

* I.D., intradermal.

Meanwhile, the three lambs exposed intradermally were sacrificed on the sixth day and showed typical severe lesions. In all, the local lesions at sites of trauma or hemorrhage were several times greater in volume than those where the inoculum had been deposited intradermally.

Discussion

In discussing these current findings, it is most convenient to consider them, in connection with earlier results, as delineating patterns of host reaction to this form of the PPLO.

Pattern of host susceptibility. It appears from this and earlier work[1,2] that goats, sheep, and swine are susceptible to infection with this form of PPLO when inoculated by parenteral routes. Similar exposures have failed to produce clinical infection in the horse, calf, dog, turkey, chicken, or small laboratory animals.

While several isolations of PPLO from caprine arthritis outbreaks have been made by this and another California laboratory,* no PPLO have been recovered from field cases of arthritis in sheep or swine. One of the PPLO found in arthritis of calves[5] appears to be innocuous to sheep. Edematous swellings and death have resulted from subcutaneous injection of the classic bovine pleuropneumonia agent into sheep and goats.[6]

Patterns of transmission. Actual parenteral injection by various routes has produced disease consistently in susceptible hosts. Only occasional infection has resulted from oral exposure or aerosol inhalation. In earlier work[1] one goat was infected by conjunctival exposure. Sheep exposed by pen contact or skin scarification have been unaffected. The demonstration of PPLO in feces and urine[2] suggests that natural transmission is by ingestion or inhalation.

Clinical pattern. A uniform clinical pattern was seen throughout this work. The incubation period after actual injection by any route did not exceed 72 hours, and signs often appeared on the day following inoculation. The lamb infected by aerosol inhalation was febrile the following day. The incubation periods for conjunctival and oral exposure were 4.5 and 6 days, respectively. The shorter incubation periods were usually followed by a septicemic form of the disease, while longer ones led to arthritis, serositis, and meningitis. Hyperthermia, deep depression and, in the more prolonged cases, marked lameness were the chief clinical signs. The disease ran a course of from 2 to 12 days in experimental animals. While most field cases died acutely, some survived as long as 5 weeks.[1] Mortality was extremely high. Only 1 equivocal recovered case was seen among 45 goats affected in the original field outbreak. Among 38 affected experimental animals only 1 peculiar individual, the ram injected intramuscularly, might be called recovered. A similar high mortality has been reported in caprine pleuropneumonia[7] and in a cellulitis produced by a related organism.[8]

Pattern of systemic lesions. Two patterns of systemic lesions, intergrading to some extent, have appeared consistently. These have correlated closely with the duration of the incubation period and the course of the disease. The more acute cases in which death occurred within 4 days showed only nonspecific evidence of septicemia. Those that died after a longer course developed synovitis, serositis, and meningitis.

In one animal or another each diarthrodial joint was involved, but the larger leg joints and the atlanto-occipital articulation were most frequently and severely affected. Affected joints showed a modest increase of turbid synovial fluid containing abundant fibrin. Mononuclear and neutrophilic leukocytes heavily infiltrated the synovial membrane. Ulceration of the articular cartilage was observed rarely.

The serous cavities showed large amounts of fibrin with variable quantities of thin turbid fluid. The serosae showed leukocytic infiltration with attached layers of fibrin containing many neutrophils.

The leptomeninges, while sometimes thickened and opaque, usually showed very little grossly. Microscopically, the pia-arachnoid was heavily infiltrated by mononuclear and neutrophilic leukocytes and occasionally showed a surface ibrin layer. The lesions were always most severe over the cerebral hemipheres.

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* M. L. Murdock, Livestock and Poultry Pathology Laboratory, Sacramento, Calif. Personal communication.

Focal hepatic necrosis, observed in both natural and experimental cases in goats, was not seen in sheep or swine.

Synovitis, often with serositis, has been reported in many natural or experimental PPLO infections, including bovine pleuropneumonia,[9,10] caprine pleuropneumonia,[7] contagious agalactia,[11] porcine polyserositis,[12] and a peritonitis in a goat.[13] Even the pulmonary form of PPLO of sheep, an organism of low virulence, has produced transient arthritis in some experimental lambs.[14]

Pattern of certain local lesions. The development of large inflammatory lesions in areas of hemorrhage or trauma distant from the sites of intradermal inoculation explains the massive neck cellulitis seen in many intravenously and intratracheally inoculated animals. The attendant tissue damage is probably more important than the quantity of PPLO contamination, since careful intradermal or subcutaneous injection produced much smaller lesions with much larger inocula. In accord with this idea is an earlier observation that cellulitis appeared in the neck of a goat after a blood sample had been drawn several days following conjunctival exposure.[1]

Both bovine[9,15] and caprine[7] PPLO produce severe local inflammatory lesions after subcutaneous or intramuscular inoculation. A naturally occurring cellulitis of this sort has been reported in goats in Greece.[16]

Some microbiological features. Examination of most acute cases showed PPLO widespread in tissues and fluids; the few cases of longer duration suggested that the bacteremia had subsided, joint infection persisting. Agglutinins appeared as early as 6 days after infection, while the bacteremia was often present. The organism was very resistant to freezing, being fully virulent after 4 years at −20° C.

Identity of this form of PPLO. Culturally, the caprine arthritis form of PPLO resembles the agent of caprine pleuropneumonia, but pneumonia has not been seen in field cases nor has it been reproduced in experimental animals. Possibly the organism is related to the cellulitis-producing Spartan PPLO, classified by Edward[17] as caprine pleuropneumonia, or to the Australian PPLO from peritonitis in a goat.[13] Pneumonia was not reported in either of these entities.

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ORGANISMS OF THE PLEUROPNEUMONIA GROUP OF AVIAN ORIGIN: THEIR CLASSIFICATION INTO SPECIES

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Respiratory infections of poultry have aroused considerable interest in recent years, particular attention being paid to the etiology of the syndromes recognized in North America as chronic respiratory disease (CRD) of fowls and as turkey sinusitis. Early work suggested that these diseases were caused by a virus (Van Roekel *et al.*, 1952); later, organisms of the pleuropneumonia group were isolated. Markham and Wong (1952) showed that, even after thirteen subcultures in artificial media, these organisms were pathogenic for chick embryos and that suspensions of the embryos reproduced catarrh in inoculated fowls. There is now an extensive literature on the relationship of the pleuropneumonialike organisms to CRD and turkey sinusitis. Although most authors have concluded that the organisms cause these diseases, doubts have been expressed. Fahey and Crawley (1954) claimed to have isolated a virus from infected fowls. Chu (1954), moreover, isolated pleuropneumonialike organisms from birds with other infections and even from normal birds.

It seemed possible that this confusion might have arisen from the fact that more than one species of the pleuropneumonia group was present in the respiratory tracts of birds, perhaps only one species being pathogenic and the others commensals. Both in man and in animals, more than one species has been isolated from the same site (Edward, 1954). Therefore, the biological and serologic properties of 40 strains isolated from the respiratory tract of fowls and turkeys have been examined. These strains had been isolated in other laboratories; the majority were received during 1955. It became apparent immediately that the strains belonged to at least three different species and this finding was quoted briefly by Freundt in his monograph in Bergey's *Manual of Determinative Bacteriology* (1957). It is now possible to report the results in detail. The organisms studied will be referred to here as organisms of the pleuropneumonia group but, if the classification of Edward and Freundt (1956) is accepted, it would be more consistent with bacteriological usage to call them Mollicutales or Mycoplasmatales (Freundt, 1957).

Material and Methods

The 40 strains examined were collected from a number of laboratories, mostly in the United States, TABLE 1. The properties of one strain, Fowl, have been reported previously (Edward, 1954), and a named species, *Mycoplasma gallinarum*, based on this strain, has been established (Edward and Freundt, 1956). It is a routine in bacteriology to purify cultures by subculture from single colonies. This was done at least once with each strain of a pleuropneumonialike organism, using a Pasteur pipette drawn out to a fine capillary point.

The media and methods employed to examine biological properties have been described previously (Edward, 1950; 1954) and only a few modifications ere introduced. Thymic nucleic acid was added routinely to the medium Edward and Fitzgerald, 1952), although it was not essential for the growth f these strains. To determine fermentative capacity, a semisolid medium o which was added 1 per cent of carbohydrate and 0.002 per cent of phenol ed was used.

The methods for preparing antisera in rabbits and for testing for agglutinaion and for inhibition of growth were those described previously (Edward and itzgerald, 1954). It should be noted that the antigens used in the serologic ests were grown in media containing horse serum, but the antigens used to noculate rabbits to prepare antisera always were grown in media enriched vith rabbit serum only. There is reason to believe, at least on theoretical rounds, that nonspecific reactions may result if antigens used to inoculate abbits are grown in the presence of horse or other foreign serum, even though such antigens are washed previously. The complement fixation tests were carried out by the plate method, using Le Bouvier's (1952) modification of the technique of Fulton and Dumbell (1949).

TABLE 1

RESULTS OF IDENTIFICATION OF STRAINS RECEIVED

Source	*M. gallinarum*	*M. iners*	*M. gallisepticum*	Glucose-fermenting strains not further classified	Total
H. E. Adler (1958)	0	0	0	3*	3
H. E. Adler (1958)	0	1	2	1	4
H. P. Chu (1958)	3	0	0	0	3
R. D. Conrad†	4	6	1	2	13
J. F. Crawley (1954)	0	0	1	0	1
J. G. Lecce‡	3	1	0	0	4
F. S. Markham (1952)	0	0	11	0	11
Sweden	1	0	0	0	1
Total	11	8	15	6	40

* One strain was not examined serologically nor tested for its ability to ferment sucrose.

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‡ University of Pennsylvania, Philadelphia, Pa.

Results

When the biological properties of the strains were examined, it quickly became apparent that they could be classified into three groups in respect to three properties. The first group (Group A), which included strain Fowl, when grown on 20 per cent horse serum agar formed a pearly film on the surface, together with minute spots in the substance, of the medium (Edward, 1954). The strains also produced hemolysis in horse-blood agar, but did not ferment glucose. These are the properties previously described for *M. gallinarum* (Edward, 1954; Edward and Freundt, 1956). The second group (Group B) did not form a film or spots on horse-serum agar, did not hemolyze horse-blood agar, and did not ferment glucose. The third group (Group C) did not form a film or spots, but did hemolyze blood agar and did ferment glucose.

Three methods were used to determine antigenic relationships, namely the agglutination reaction, the complement fixation test, and a test for inhibition of growth (Edward and Fitzgerald, 1954). Many of the strains were examined by all three methods. An antiserum prepared in a rabbit against strain Fowl agglutinated the 10 strains classified in Group A. It also fixed complement with the 9 strains tested. Growth of 3 strains was inhibited completely; growth of 2 other strains was inhibited partially; and 5 strains grew normally in the presence of the antiserum, TABLE 2. The variable result with the inhibition test may have been due to the low inhibitory titer of the antiserum or to minor antigenic differences between strains that were revealed by this test, but not by the agglutination reaction. The reverse finding has been noted previously with *M. hominis*, where serologic differences in the agglutination test were not found in the inhibition test (Edward and Fitzgerald, 1954). None of the avian strains in the other two groups with different biological properties reacted with the Fowl antiserum. The strains in Group A thus appear closely to resemble strain Fowl, which is the representative strain of *M. gallinarum*, and they must be considered as belonging to this already established species.

TABLE 2
RESULTS OF THE SEROLOGIC TESTS

Group A				Group B				Group C			
Fowl antiserum				M antiserum				X95 antiserum			
Strain	Agglut. titer	CFT tite	Inhibition	Strain	Agglut. titer	CFT titer	Inhibition	Strain	Agglut. titer	CFT titer	Inhibition
168	64	16	−	4	512	32	+	X119	32	8	+
j	32	8	+	583	256		+	X153			+
o	64	16	±	624	256		+	X146	64	16	+
a	64	16	±	671	128	32	+	1136	16		+
P583	64	16	+	1054	128		+	1027	32	32	+
853	128	8	+	OZG	128		+	FG	64		+
1006	32	32	−					S6G	64		+
T105	64	16	−								
190	32	32	−								
17	32		−								

An antiserum was prepared against strain M, one of the Group B strains. It inhibited the growth of all the other strains classified in this group; it also agglutinated the six strains tested in this group and fixed complement with the two strains tested (TABLE 2). It did not react with any of the other avian strains. Thus, it would appear that the Group B strains differ sufficiently from *M. gallinarum* in their biological and serologic properties for them to be regarded as a new and distinct species of the pleuropneumonia group. Since they reacted negatively in the three biological tests used to differentiate them from the other avian strains, the name *M. iners* nov. sp. is suggested for the species, strain M being the representative strain. Strains of this species produced relatively small colonies that were otherwise typical of organisms of the pleuropneumonia group. They did not grow on the basal medium alone without enrichment with serum. They grew as well on media enriched with rabbit serum as on media containing horse serum. In semisolid media they grew throughout the medium, producing a smooth growth.

A third antiserum was prepared against strain X95, a Group C strain. It inhibited the growth of 14 glucose-fermenting strains. Of these 14 strains, it also agglutinated the 6 tested and fixed complement with the 3 tested (TABLE 2). One glucose-fermenting strain was not tested against this antiserum; the remaining 5 strains did not react with the antiserum in any of the 3 serologic tests, with the exception that 1 strain only fixed complement with the antiserum. None of the avian strains belonging to the other groups reacted with this antiserum. It would thus appear that strain X95 and the 14 strains antigenically similar to it differ sufficiently in their biological and serologic properties from strains of the 2 species already designated to merit classification in a third species. It is proposed that this species should be named *M. gallisepticum* nov. sp., the representative strain being X95.

Growth of strains of this species was highly characteristic. Even when the plates were inoculated heavily, other members of the pleuropneumonia group produced discrete colonies, at least in regard to their central spots. The growth of *M. gallisepticum*, on the other hand, tended to become confluent with piling up of the central growth; central spots were slightly evident. Separated individual colonies varied in size, but otherwise had no special features, being typical of the pleuropneumonia group. There was no growth on the basal medium without serum. Strains grew well on media enriched with rabbit serum, possibly slightly better than with horse serum. In semisolid media there was smooth growth throughout the medium. In addition to glucose, there was fermentation of maltose, mannose, dextrin, starch, and glycogen. The fermentation of fructose was variable. Galactose, lactose, sucrose, mannitol, and dulcitol were not fermented.

Of the remaining 6 strains, 1 was lost before it could be examined serologically; the other 5 strains could not be classified in any of the 3 species. The 5 strains originally had been placed in Group C, but were found later to differ from *M. gallisepticum* in their fermentative properties. Like strains of that species, they fermented glucose, maltose, dextrin, starch, and glycogen and did not ferment lactose, galactose, mannitol, and dulcitol. They differed in fermenting sucrose and in not fermenting fructose and mannose. An antiserum was prepared against one of these strains (15804) but, although this serum had a high titer against the homologous organism, it did not react with any of the other avian strains. The results of cross-tests between this serum and the other 3 antisera and their corresponding antigens are shown in TABLE 3. No attempt has been made to classify these 5 strains further and their position is still uncertain. They may represent serologic types of *M. gallisepticum* or it may be that they should be regarded as belonging to one or more new species.

Discussion

In TABLE 1 the strains are listed according to the laboratory from which they were received. Of the 40 strains, 11 were identified as *M. gallinarum*, 8 as *M. iners*, and 15 as *M. gallisepticum*, there being an additional 6 glucose-fermenting strains that were not classified. The numbers of strains of each species identified in no way reflects the relative incidence of these species in fowls and turkeys. It is, however, demonstrated that laboratories studying the relationship of organisms of the pleuropneumonia group to respiratory infection of birds have been working with organisms belonging to several different species. Only 2 laboratories sent more than a few strains. It is of interest that all 11 strains from F. S. Markham were identified as *M. gallisepticum* whereas, of 13 strains from R. D. Conrad, 4 were identified as *M. gallinarum*, 6 as *M. iners*, 1 as *M. gallisepticum*, and 2 unclassified glucose-fermenting strains.

TABLE 3
RESULTS OF RECIPROCAL TESTS OF ANTISERA AND THEIR CORRESPONDING ANTIGENS

Strain	Fowl antiserum			M antiserum			X95 antiserum			15804 antiserum		
	Agglut. titer	CFT titer	Inhibition	Agglut. titer	CFT titer	Inhibition	Agglut. titer	CFT titer	Inhibition	Agglut. titer	CFT titer	Inhibition
Fowl	128	32	+	<16	<2	–	<16	<2	–	<16	<2	–
M	<16	<2	–	512	32	+	<16	<2	–	<16	<2	–
X95	<16	<2	–	<16	<2	–	64	32	+	<16	<2	–
15804	<16	<2	–	<16	<2	–	<16	<2	–	512	32	+

Since this work started, Adler and his colleagues have published the results of investigations along similar lines. In addition, they have tested strains characterized by their biological and serologic properties for their pathogenicity for birds (Yamamoto and Adler, 1958*a*, *b*). The strains examined belonged to one or another of 5 distinct antigenic types (Groups I to V). Four of the type strains were supplied by Adler. Strains S6 and F, belonging to Group I, were identified as *M. gallisepticum* and strain O (Group IV) as *M. iners*. Strain Tu (Group II), which fermented carbohydrates, did not react with any of the antisera. The finding of Yamamoto and Adler that this strain fermented sucrose was confirmed, and it is of interest that these avian strains are the only organisms of the pleuropneumonia group that as yet have been found to ferment this carbohydrate.

The work of Yamamoto and Adler, following upon that of Markham and Wong (1952) strongly suggested that *M. gallisepticum* is pathogenic and capable of infecting the respiratory tract of fowls and turkeys. Strain O, in the same set of experiments, was found not to be pathogenic, and *M. iners* would thus appear to be a commensal. It would also seem from the observations of Chu (1958) that *M. gallinarum* is a commensal. The organisms described by Chu as "coccobacilliform bodies" and regarded as a likely cause of respiratory disease are probably *M. gallisepticum*.

It would appear that the confusion that existed for a number of years in egard to the relation of organisms of the pleuropneumonia group to avian nfection was due largely to a failure to study the organisms sufficiently. In bacteriology it is established practice to submit any bacterium isolated to such examinations as are necessary to identify and classify it in a taxonomic system. It is equally important that the same practice be adopted when an organism of the pleuropneumonia group is isolated. To repeat the advice given previously (Edward, 1950): "In the examination of material it is therefore necessary not only to determine that a pleuropneumonialike organism is present but to identify the species." Determination of both biological and serologic properties permits the classification of strains into groups, even if it is considered premature to establish new species.

Summary

A study has been made of the biological and serologic properties of 40 strains of organisms of the pleuropneumonia group obtained from other laboratories investigating the relation of these organisms to respiratory infection of fowls and turkeys.

Eleven strains were identified as *M. gallinarum.*

Another 8 strains, whose biological and serologic properties were the same but differed from the other strains, were regarded as belonging to a new species, *M. iners.*

Fifteen additional strains, also with distinct biological and serologic properties, were regarded as a third species, *M. gallisepticum.*

The examination of 1 strain was not completed. The remaining 5 strains could not be included in the 3 species and were not classified further.

These results emphasize the importance of identifying any organism of the pleuropneumonia group that is isolated.

Acknowledgments

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MYCOPLASMA, THE CAUSE OF CHRONIC RESPIRATORY DISEASE

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The specific disease that *Mycoplasma* causes in the field has been difficult to demonstrate in the laboratory. Similarly, bovine pleuropneumonia, although caused by a highly pathogenic organism, was not reproduced experimentally for many years.[1,2] Pathogenicity studies of *Mycoplasma* in fowl had little early success.[3-6] In 1935, Nelson[7] described in chickens a coryza caused by coccobacilliform organisms—a *Mycoplasma*, according to Smith *et al.*[8] For about twenty years after Nelson's studies, the specific role of *Mycoplasma* in in respiratory diseases of fowl was unknown.

In 1952, Markham and Wong[9] showed the pathogenicity of a known *Mycoplasma* for turkeys, yet considerable confusion remained as to the role of these organisms in field cases of chronic respiratory disease (CRD). In many investigations *Mycoplasma* cultures produced a mild disease or none at all. The etiological picture was complicated further by field evidence implying that the production of a serious disease with *Mycoplasma* isolated from fowl requires the presence of other factors or infection. Newcastle disease and infectious bronchitis, as well as other commonly known respiratory disease viruses, were each capable of inciting more severe disease than any *Mycoplasma* infection alone.[10,11] Fahey and Crawley[12] described a new virus as a synergist necessary if more than mild CRD is to be produced in chickens. Fahey[13] later found an unidentified virus associated with infectious sinusitis of turkeys. According to Fahey, this virus, together with *Mycoplasma*, was responsible for severe CRD of turkeys. In chickens, *Hemophilus gallinarum* and *Mycoplasma* in combination have been shown to produce more severe coryza than either organism alone.[14] As with other animals, there is not much question that *Mycoplasma* produces a more severe disease when acting in conjunction with other infections. However, uncomplicated *Mycoplasma* infections do occur in domestic fowl. Failure to produce the extensive air sacculitis seen in *Mycoplasma*-infected field cases may have been due to small numbers of pathogenic *Mycoplasma* in the inocula. Recent papers by Adler *et al.*[15] and Yamamoto and Adler[16] reported that chicken and turkey tissues usually contain *Mycoplasma* of varied pathogenicity. The procedures commonly used to isolate *Mycoplasma* from avian tissues favor the growth of strains of reduced pathogenicity. In tests, the majority of these strains failed to produce disease in turkeys. Strains of known pathogenicity readily reproduced many of the characteristics of CRD of chickens and turkeys, but not all forms of so-called air-sac infection could be induced. Imprecise naming of the disease added to the confusion. For some time, two terms—CRD and air-sac disease—were used interchangeably for the disease in the field. Van Roekel[17] suggested using only CRD for the definite poultry disease entity caused by *Mycoplasma*, reserving the name air-sac disease for indefinite pathological processes caused by a number of infectious agents that involve the air sacs.

During the last few years, a number of papers have reported that *Mycoplasma* cultures revert to bacteria.[18-20] This paper deals with L forms of bacteria versus *Mycoplasma* as a cause of CRD as studied by serial propagation *in ovo*, *in vitro*, and *in vivo*.

Pleuropneumonialike organisms (PPLO) recently have been placed in the genus *Mycoplasma*.[21] Only one species has been described for chickens and turkeys: *M. gallinarum*. Its description in the seventh edition of Bergey's *Manual*, however, does not conform to the characteristics of the known pathogens of this genus. To simplify future nomenclature, PPLO are herein referred to as *Mycoplasma*, without reference to species name.

Methods

Strains of Mycoplasma. Six strains of *Mycoplasma* isolated from chickens and turkeys were studied: (1) strain SA, a nonpathogenic strain isolated from a turkey with infectious sinusitis; (2) strain Tu, a nonpathogenic strain isolated from the turbinate of a chicken; (3) strain O, isolated from the pericardial sac of a chicken with CRD and the cause of arthritis in chicken embryos, but nonpathogenic to adult chickens or poults; (4) strain S6, isolated from the brain of a turkey, and pathogenic to turkeys and chickens on inoculation by various routes; (5) strain PH, a pathogenic egg-adapted organism isolated from turkeys with infectious sinusitis, the only strain that fails to grow on a variety of enriched artificial media; and (6) strain F, isolated from the trachea of a chicken showing CRD, and virulent to chickens and turkeys.

Comparative observations were made on the various *Mycoplasma*, on L phase of *Proteus*, and of a *Streptococcus* provided by L. Dienes.

Media. For all experiments with *Mycoplasma* the basal media were Bacto-PPLO broth and Bacto-PPLO agar enriched with 1 per cent yeast hydrolyzate and 10 per cent serum. Before horse serum was added, the *p*H of the broth medium was adjusted to either 7.0 or 7.8. The L form of *Proteus* was grown on the same enriched Bacto-PPLO agar medium, with further additions of 3 per cent NaCl and 5000 U. of penicillin per milliliter. The L-phase *Streptococcus* was propagated on the same enriched agar, with only NaCl added.

Experimental birds and eggs. One-day-old single-comb White Leghorn chickens were obtained from the University of California Department of Poultry Husbandry at Berkeley. The source flock had been found free of known respiratory infections and had not been vaccinated against any disease. Day-old poults also were obtained from flocks free of infectious sinusitis. Fertile chicken eggs came from the flock of the Department of Poultry Pathology at Davis, Calif. There was no active CRD in this flock, although sera agglutinins for S6-type *Mycoplasma* were present.

Experimental

Morphologic characteristics. Since it has been shown by Ørskov,[22] Edward,[23] and Freundt[24] that colonies of L-phase variants can be distinguished from colonies of *Mycoplasma*, the following study was made of the comparative morphology of known L forms and the five strains, previously listed, that would grow on artifical media.

Five strains of *Mycoplasma* from a tenth-culture passage in noninhibitory roth were streaked on enriched agar. After 48 hours of incubation at 38° C, he colonies were fixed and stained by the method of Klieneberger-Nobel.[25] or comparison, the two L forms of bacteria were propagated for the same eriod on specially prepared agar.

There was a considerable difference in colonial appearance between the two acterial L-phase organisms and the *Mycoplasma*. L-phase colonies were uch larger and thicker than *Mycoplasma* colonies, and were readily visible acroscopically. They were quite similar to small bacterial colonies. Deailed observation through a dissection microscope at 108 diameters' magnification showed that the L-phase colonies had irregular and rather coarse surfaces, n contrast to the smooth, round, clear *Mycoplasma* colonies. Preparations of he L-phase and *Mycoplasma* colonies fixed and stained by the procedure of lieneberger-Nobel emphasized the differences. The thickness and size of the -phase colonies made it difficult to study the entire colony with this procelure. The stained L-form colonies were much coarser than *Mycoplasma* coloies, with a greater tendency to produce large bodies and other protoplastic asses. Serial passages of the *Mycoplasma* cultures from broth to agar did ot alter colony morphology. The L forms of bacteria were very unstable n broth culture, either stopping growth or reverting to parent bacteria. owever, L forms that did grow on agar from the first broth passage were orphologically unchanged.

It has been demonstrated that L-phase organisms are difficult to maintain n noninhibitory broth and have a tendency to revert to parent bacteria after roth passages. It has been shown repeatedly that *Mycoplasma* are readily ropagated serially in broth, but whether all *Mycoplasma* cultures have absoute stability in such media is open to question.

To test the stability of the *Mycoplasma* strains used in the study reported ere, the cultures described were subjected to a series of multiple transfers in iquid media from agar. All strains were propagated five times serially on agar plates by streaking from obvious *Mycoplasma* colonies to fresh agar. The same colonial types were observed at each subculture.

Agar blocks from the fifth passage were seeded into 3 separate sets of serum broth tubes, 2 sets adjusted to *p*H 7.8, and the third to *p*H 7.0. Serial passages were made at 3-day intervals by 2 individuals. The broths were periodically streaked on serum agar plates and examined 3 days later. Samples of the broth cultures were stained by the Giemsa method to observe the various forms of *Mycoplasma* and bacteria.

On the ninth passage of strain S6, a *Streptococcus* appeared in one of the broth cultures from the *p*H 7.8 series. The eighth passage was free of bacteria, and serial passage was continued from this culture. Examination of the remaining 84 tubes of uninoculated broth, from the same batch of medium that had been used for all of the subcultures, revealed a similar *Streptococcus* in 1 tube. During the eighteenth passage of the F strain, a *Streptococcus* was again encountered in a tube. This organism was retained for later pathogenicity studies. The 2 replicate passages of the F strain were negative and were propagated until the twentieth passage. At this time, an S6 strain that earlier yielded a *Streptococcus* now had a *Micrococcus.* The *Micrococcus* culture also was retained for pathogenicity studies.

As a further test of the stability of *Mycoplasma* in culture media, the procedure of Smith *et al.*[20] was applied to a typical pathogenic strain (S6) and a typical nonpathogenic strain (Tu). From 100 tubes seeded with agar blocks that had been streaked with S6 culture, 1 *Streptococcus*, 7 *Micrococci*, and a diphtheroid were obtained, in addition to S6 *Mycoplasma*, which was present in every tube. Of 100 control tubes seeded with uninoculated agar, 4 yielded *Micrococci* and none yielded *Mycoplasma.* The 100 tubes seeded with agar blocks from the Tu culture yielded a diphtheroid in addition to the Tu strain.

Work by Dienes[26] and others has indicated that metabolic reactions are similar in L-phase and parent bacteria. The *Streptococcus* and the *Micrococci* differed from the S6 *Mycoplasma* in biochemical reactions. The 2 diphtheroids failed to use any of the sugars tested, whereas the Tu and S6 strains had definite fermentation patterns, as previously described.[15]

From the foregoing studies and previous work, the cultural criteria for the S6 strain of *Mycoplasma* are found to be: (1) growth occurs only in media containing enrichments such as serum or serumlike protein; (2) growth in broth is usually uniform and slightly turbid, with no granular sediment after 3 to 6 days of incubation at 37° C.; (3) culture in broth is composed of numerous coccoid bodies (125 to 500 mμ), with occasional rings made visible in Giemsa-stained preparations, but not in the usual aniline stain; (4) small circular colonies (not exceeding 0.5 mm.) grow into the agar and consist of fine coccoid forms in a lacy network at the periphery, usually with a central dense papilla; and (5) the small typical colonies remain unchanged through many subcultures in noninhibitory media.

Pathogenicity. The *Micrococcus*, *Streptococcus*, and diphtheroid that were isolated from the *Mycoplasma* cultures, as well as the original *Mycoplasma*, were inoculated into 6-week-old poults, 5 for each culture. Prior disease was not evident, and agglutinins for the original *Mycoplasma* were absent. The S6 *Mycoplasma* from the twentieth culture passage produced severe disease in all inoculated turkeys, and agglutinins for this strain were present. Contact controls in the same pen acquired the infection. An S6-type organism was isolated on serum agar directly from the air sac. No bacterial colonies were seen on this medium. Tissue sections of air-sac lesions stained by the Giemsa method revealed the microscopic pathology characteristic of *Mycoplasma* infection,[27] and no bacteria were visible in these lesions.

Another pathogenic strain (F) was selected from a serial culture group. The twentieth culture passage of this strain was inoculated into the air sacs and infraorbital sinuses of thirteen 10-week-old poults. Eleven of them developed agglutinins for the homologous-type strain (S6 antigen). These birds were all severely diseased. Tissue sections of lesions from the affected birds showed typical *Mycoplasma* reactions and the absence of bacteria.

To determine whether these organisms could produce disease in a more resistant host, an eighteenth culture passage of the F strain was inoculated into 10 twelve-week-old cockerels via abdominal air sacs and into ten via the brain. None showed signs of the disease but, at necropsy, 5 of the birds inoculated via abdominal air sacs exhibited severe aerocystitis. Three of these ockerels had swollen livers, which histological examination revealed to be the ymphofollicular reaction described for this infection.

Mycoplasma were isolated from the air-sac lesions on noninhibitory media. Sections stained by the Giemsa method did not show bacteria, and the pathology was characteristic of CRD. The birds that had been inoculated intracerebrally showed no signs of disease, although a moderate vasculitis[28] was observed in tissue sections of the brain, and the organism was isolated readily rom brain tissue.

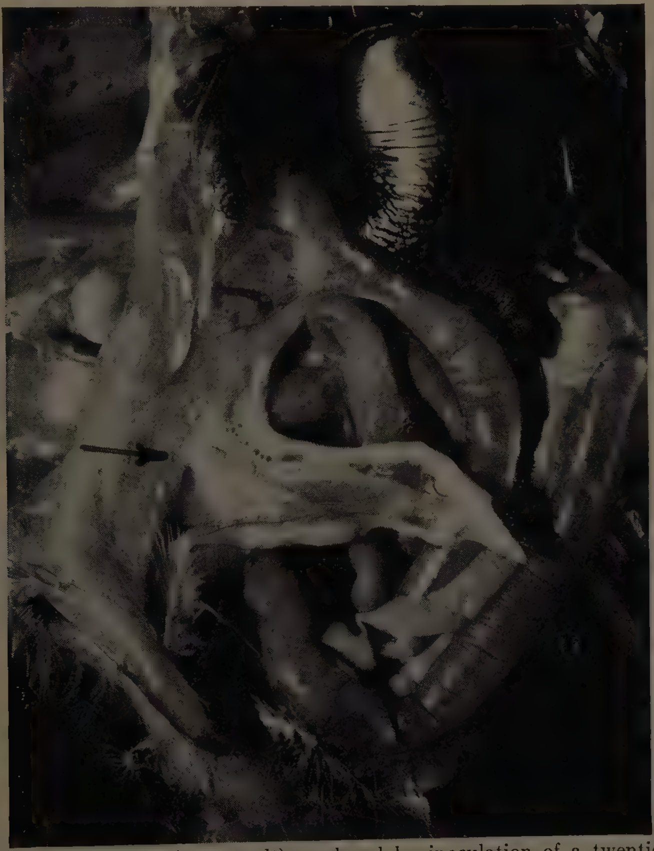

FIGURE 1. Air-sac lesion (in a poult) produced by inoculation of a twentieth-passage culture of *Mycoplasma* (S6).

A third pathogenic strain (PH) that did not grow in artificial media was propagated without inhibitors through 8 passages in chicken egg embryos and then inoculated into turkeys: 5 each via the sinus, air sac, and brain. All of the poults developed agglutinins for the S6 antigen, but showed no signs of brain involvement. *Mycoplasma* from sinus exudate grew profusely in a noninhibitory chicken embryo tissue culture, but failed to grow in broth. A few colonies were observed in agar seeded from the tissue culture.

To enhance pathogenicity and test L reversion in embryos, the so-called nonpathogenic strains Tu and O were propagated in chicken embryos through 9 and 11 passages, respectively. No reversion to bacteria took place, and uniform mortality began the fifth day after inoculation. Inoculation of these strains into the sinuses and abdominal air sacs of poults failed to produce lesions of disease. There were no agglutinins for the S6-type antigen or the antigen homologous for the *Mycoplasma* strains inoculated.

In another test, yolks taken from embryos infected with the SA strain, which heretofore had been entirely nonpathogenic when inoculated into the air sacs and sinuses of 12 eight-week-old turkeys, produced a mild aerosacculitis, but without sinusitis. These birds had agglutinins for the SA strain and no agglutinins for the S6-type antigen.

Cultures or embryonating eggs usually had approximately 10^8 organisms per milliliter of material. The question arose whether, possibly, lesions and serologic response were produced only by overwhelming inoculation of *Mycoplasma.* Accordingly, broth from a fifth culture passage of the S6 strain was diluted to 10^{-8}. The diluted organisms were inoculated into abdominal air sacs of 10-week-old turkeys, 5 birds per dilution. At a dilution of 10^{-8}, 3 of the 5 birds developed severe aerocystitis. At all higher concentrations, all birds developed lesions. A similar experiment, on cockerels, was conducted a month later, using 7 birds for each dilution. These birds were inoculated with the eighteenth culture passage of the S6 strain. Each milliliter of undiluted culture contained approximately 10^9 organisms. At a dilution of 10^{-6}, 5 birds of 7 developed a moderate to severe aerocystitis. The majority of the cockerels inoculated with broth at the lower dilutions developed severe disease of the air sacs. At each dilution, there was a definite correlation between the presence of lesions and the presence of agglutinins for the homologous *Mycoplasma* antigen.

Discussion

In determining the etiology of CRD it was necessary to show that organisms associated with the condition would produce the disease consistently in the absence of other infectious agents. The agent most often found in the tissues of birds with CRD is a species of *Mycoplasma.* Since it has been suggested that some *Mycoplasma* may be an L form of bacteria, this possibility was tested. Five *Mycoplasma* strains were tested for purity by morphologic, cultural, and pathogenicity studies.

The strains of *Mycoplasma* and two known L forms differed considerably in morphologic features. In all passages *Mycoplasma* colonies were much more delicate than L-form colonies. Furthermore, the more rapid growth and the development of large protoplastic masses in L forms were also useful in distinguishing it from *Mycoplasma.* There were definite morphologic differences between the two growth forms, but these characteristics could not be relied upon for complete differentiation. The so-called nonpathogenic strains can be differentiated on the basis of colonial morphology and growth rate. Colonial characteristics may be used to make a rapid presumptive distinction between *Mycoplasma* strains and L forms of bacteria.

A number of papers have dealt with reversion in broth cultures by so-called

Iycoplasma* to bacteria.[20,29,30] Most of the reports were concerned with
rganisms that had been recovered from cases of nonspecific urethritis of
ıan. In almost every instance the "*Mycoplasma*" proved to be an L form of
Corynebacterium. Recently, Kelton and Gentry[19] reported that 20 cultures
f avian *Mycoplasma* from a number of laboratories reverted to bacteria,
sually by the third transfer in noninhibitory broth. A *Corynebacterium* was
ound in 1 of the 20 cultures. Two strains reverted to *Streptococci*, and 17
trains to *Micrococci*. These researchers pointed out that the use of penicillin
n culturing air-sac and tracheal exudates favored the development of L forms.
They did not determine the pathogenicity of the parent bacteria for chickens
nd turkeys.

In present tests for any relationship to the L phase of bacteria, 20 serial
assages of both pathogenic and nonpathogenic *Mycoplasma* strains were
nade in triplicate in noninhibitory media. Bacteria were encountered in
ome passages, but they were not consistently of the same genera in any single
ulture. Furthermore, the bacteria differed appreciably from the original *My-*
oplasma in fermentation reactions and, in addition, were not pathogenic for
urkeys. It would be unusual for an L form to be more virulent than the
arent bacterium. The bacteria found in the *Mycoplasma* cultures were evi-
lently contaminants encountered in serial culture, using serum-enriched media.
Attempts to convert the *Mycoplasma* to L form by the method of Smith *et al.*[20]
ailed. The application of this procedure emphasized again that contamina-
ion of cultures can occur readily when many tubes of culture media are used
o determine the probability of reversion.

The major question of *Mycoplasma* reversion to bacteria seems to be con-
erned with organisms isolated from genital infections of man. The role of
hese organisms in these diseases seems to be unknown. Smith *et al.*[20] reported
hat the Campo strain of *Mycoplasma* reverted to a diphtheroid. These re-
sults indicate an antigenic relationship between the bacteria and the *Myco-*
plasma, although there seemed to be a greater antigenic similarity between
another diphtheroid and the *Mycoplasma*. This suggests an interrelationship
of the organisms found in the urethra of man. Earlier, Keller and Morton[31]
were unable to demonstrate any pathological manifestations from inoculation
of the Campo strain of *Mycoplasma* in embryonating chicken eggs. However,
he absence of embryo mortality may have been due partly to the fact that
noculation was by the allantoic route; moreover, the organism used had been
dapted to grow in artificial media. It has been our experience with *Myco-*
plasma isolated from poultry that the yolk sac route of inoculation is most
ethal for the embryo. Yamamoto and Adler[16] showed that some *Mycoplasma*
strains of low virulence grew profusely in embryos without causing mortality.
Mycoplasma strains pathogenic to mammals were lethal to chicken embryos.
Sheriff and Piercy[32] found that the bovine form of PPLO is most lethal for
embryos when inoculated through the yolk sac. The only *Mycoplasma* lethal
by all inoculation routes was a highly virulent organism isolated from goats.[33]
It would be interesting to study *Mycoplasma* of human origin for pathogenicity
by yolk-sac inoculation after embryo adaptation. Under such conditions the
Campo strain would perhaps revert to a diphtheroid and kill embryos.

Two *Mycoplasma* of avian origin did not revert in embryos in the absence of antibiotics, although embryos had been killed by the organisms. One of the *Mycoplasma* cultures used in the present study also failed to revert to a bacterium on serial embryo passage, although it was lethal. Recently, Wittler *et al.*,[29] using HeLa tissue culture, reverted a *Mycoplasma* to an L form, and the L form to a *Corynebacterium*. Under specific conditions the *Mycoplasma* were cytopathogenic. Tissue culture may offer a sensitive procedure for studying the pathogenicity of *Mycoplasma* and L forms. It is unfortunate that the role of *Mycoplasma* in infections of the human urethra is not known, since these organisms have been studied in detail for L reversion. Fortunately, there are *Mycoplasma* of avian origin that are pathogenic for chickens and turkeys.

In the present experiments the pathogenic strains produced disease even after the cultures had undergone serial passage 20 times in artificial media. After such passage dilution it is unlikely that viruses or other organisms were present in this inoculum. The specific *Mycoplasma* was recovered from the diseased air sac, and bacteria were absent, as established both by culture and by examination of Giemsa-stained tissue sections. The majority of pathogenicity studies of L-phase bacteria has shown reductions in pathogenicity in transfer. Freundt[34] clearly demonstrated that the L form of *Streptobacillus moniliformis* was only slightly pathogenic for mice. As long as the organism was maintained in the L form, disease did not result. Reversion to the bacillary form took place readily in the host, and many mice consequently died of septicemia. Silberstein[35] showed that *Salmonella typhimurium* L phase was not pathogenic in that form, and that reversion to the bacillary form occurred in the mice, with accompanying pathogenicity. Schauder had a similar experience with *S. typhimurium* L forms that were fed to mice. A lag in development of the disease corresponded to the time necessary for the organism to revert to the bacillary form.

From the L phase of *Vibrio comma* Tulasne and Lavillaureix[36] derived an endotoxic substance that was lethal to mice. The toxic substance was stable at 100° C. after treatment with trichloroacetic acid. The *Mycoplasma* of avian origin produced disease in turkeys at a dilution of 10^{-8} of the culture. The lesions of the air sacs of the birds affected with very few organisms were comparable to the gross pathology at the lower dilution. The lesions occurred within 10 to 21 days. These observations suggest that the disease was associated with the growth of the organisms rather than with an endotoxic substance per se. The turkey seems to provide an ideal environment for the growth of pathogenic *Mycoplasma*, yet no reversion took place. The pathological significance of the other so-called nonpathogenic *Mycoplasma* is not clear. Further work must be done to determine their role in CRD and other diseases of poultry. The pathogenic *Mycoplasma* did not revert to bacteria in affected chickens and turkeys exhibiting aerocystitis, sinusitis, and other gross and microscopic lesions of chronic respiratory disease. If these strains of *Mycoplasma* are an L phase of bacteria, they are in a well-fixed state. At present, adoption of a proposal that these *Mycoplasma* be classified as an L phase of bacteria would cloud the understanding of the cause of CRD.

Summary

The *Mycoplasma* associated with chronic respiratory disease are not reverted o bacteria during repeated passage in culture media, chicken embryonating ggs, and birds. The organisms are capable of producing CRD in chickens nd turkeys after a number of culture passages and after a high dilution of the rganisms (10^{-6} to 10^{-8}).

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EXPERIMENTAL REPRODUCTION OF CHRONIC RESPIRATORY DISEASE

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Chronic respiratory disease (CRD) is probably as much a problem today as it was when first reported by Delaplane and Stuart[1] in 1943. Although the theory concerning the etiology has changed from that of a virus[1] to the pleuropneumonialike organisms (PPLO),[2] the problem of artificial reproduction of the disease has remained. Without a reliable laboratory method for duplication of the disease syndrome, it has been difficult to determine the value of therapeutic control measures.

Inasmuch as the taxonomic and physiological status of PPLO has remained confused for some years, it has been difficult to ascertain the pathogenic mechanism of the organisms. Evidence presented by Gross[3] indicates that *Escherichia coli* is a complicating factor in the pathogenic picture of CRD. This paper presents a further elaboration of an organism, apparently unrelated to the disease, and its possible synergistic or stressing action with a PPLO toward the causation of experimentally reproduced CRD.

Several procedures were utilized to infect birds with CRD: intraperitoneal injection and aerosol inhalation of the organisms. In order to eliminate, as much as possible, stress factors from earlier vaccination, birds that had not been vaccinated were employed. In addition, only birds that were negative for CRD antibodies, as determined by the plate agglutination test,[4] and birds from a representative group demonstrating clear air sacs were selected for the experiment. Apparently, then, within practical limits, the flock used was essentially free of clinical CRD and PPLO infection.

INTRAPERITONEAL PROCEDURE

Materials and Methods

White Leghorns, mixed sexes, ten weeks old, were divided into 4 groups of 100 birds. A PPLO (No. A5969) strain was standardized by adjusting the culture to obtain a hemagglutination titer of 1 to 16. An encapsulated *Klebsiella pneumoniae* culture isolated from swine and standardized after 18 hours' growth to a lumetron reading allowing 40 per cent light transmission through the green filter (No. 530) was used for synergistic purposes.

Group I consisted of the noninoculated control birds. Group II received 1 cc. of the PPLO suspension for a total of 3 inoculations spaced 48 hours apart, intraperitoneally at a site approximately 1½ in. to the left and slightly forward of the vent. Group III received 1 cc. of the *Klebsiella* suspension in the same manner as Group II. Group IV received 1 cc. of the PPLO culture and 1 cc. of the *Klebsiella* culture concurrently in the same manner as Groups II and III. All 4 groups were separated from each other in isolation pens approximately 100 feet apart and kept under observation for 6 weeks.

Results

Group I (*noninoculated control*). These birds remained in excellent condition throughout the trial period. Air sacs remained clear, and feed and water consumption was normal. The birds were negative for PPLO antibodies at the end of the experiment. In addition, PPLO was not recovered after culturing the tissues.

Group II (*PPLO*). Outward clinical symptoms were not observed. The birds followed a normal course in growth and feed and water consumption patterns. However, 75 per cent of this group manifested a moderate amount of air-sac involvement, both thoracically and abdominally, as determined by post-mortem examination. Postinoculation bleedings were positive for CRD. The agglutination particles, however, were moderate in size. PPLO was recovered after culturing air sacs in 63 per cent of these birds.

Group III (*Klebsiella alone*). This group likewise did not demonstrate any outward clinical respiratory symptoms. However, grossly these birds appeared to be less active for several weeks after the last inoculation. Air sacs showed clear membranes, and there was no pericardium or lung involvement. Feed and water consumption was normal. Postinoculation bleedings were negative for PPLO antibodies, while 73 per cent of this group was positive for *Klebsiella* antibodies. Neither the PPLO nor the *Klebsiella* organisms were recovered following bacteriological examination of the organs.

Group IV (*PPLO and Klebsiella test group*). Within 1 week after the last injection, birds in this group demonstrated respiratory embarrassment characterized by coughing and sneezing. Feed and water consumption was below normal, and the birds were emaciated. Post-mortem examination revealed that 94 per cent of the birds possessed severe thoracic and abdominal air-sac involvement. Typical cheeselike exudate was seen adhering to the membranes, and the livers were covered with a slimy white film. Pericarditis also was observed in most of these birds. Postinoculation bleedings resulted in very pronounced CRD agglutinating particles in 98 per cent of the birds, while only 22 per cent were weakly positive for *Klebsiella* antibodies. PPLO were recovered after culturing the air sacs and livers, while the *Klebsiella* culture was recovered in only a few instances.

Although the intraperitoneal injection route proved to be very satisfactory for the experimental reproduction of CRD, it invites the criticism that the method is a highly artificial procedure. Therefore it was decided to attempt to acquire the same results through an aerosol inhalation procedure, inasmuch as the disease probably is transmitted in this manner and this is a more natural route of infection.

AEROSOL INHALATION PROCEDURE

Materials and Methods

The same organisms, standardized and used in the intraperitoneal procedure, were utilized. A nebulizer attached to an air pump exerting a pressure of 20 psi was used to spray the cultures. Four groups of 100 White Leghorns, mixed sexes, 10 weeks old, that proved negative for CRD antibodies and PPLO were used in this phase of the experiment. The birds were placed in solation pens separated approximately 100 feet from each other. Group I as the noninoculated control, Group II received the PPLO culture alone, roup III received the *Klebsiella* culture alone, and Group IV received a ombination of the PPLO and *Klebsiella* cultures.

All birds were subjected to the respective organisms in the following manner. he birds were placed in a sealed paper carton 2.5 by 2 feet with a hole at one nd just large enough for the neck of the nebulizer to enter. Each group was prayed with the respective culture under 20 lb. pressure for 5 min. with a otal of 1.5 cc. of suspension. This procedure was repeated three times at 8-hour intervals. This method ensured inhalation of the organisms due to he build-up of carbon dioxide which served as a respiratory stimulant. Furhermore, because of the confinement, dissemination of the cultures was preented. After the spraying was completed, the carton was kept sealed until he mist within had settled, when the birds were removed. Calculations stimated that each group was sprayed with 500,000 particles of the individual ultures per spraying period. The birds were kept under observation for 6 eeks.

Results

Groups I and III were still negative for PPLO antibodies 6 weeks after the ast spraying. Birds continued drinking well, and feed consumption was at he normal level. Group II manifested weakly agglutinating PPLO antiodies. Slight clouding of the air sacs was observed. Feed and water consumption was normal. PPLO were recovered from 65 per cent of these birds. Birds in Group IV were strongly positive to PPLO antibodies 6 weeks after the ast spraying. The birds were thinner, feed and water consumption had decreased, coughing and sneezing were noted, and exudate was observed adhering to the abdominal and thoracic air sacs. PPLO were recovered from the membranes of 90 per cent of these birds. *Klebsiella* antibodies were detected in only 3 per cent of this group, and the organism was not recovered in any instance.

Since vaccination procedures apparently can serve as a stress factor toward the development of CRD, it was decided to repeat the experiment with the additive stress of laryngotracheitis virus. This phase of the experiment served as a control from the standpoint of determining whether the subjection of the birds to the synergistic or stressing action of the laryngotracheitis virus would duplicate the activity of the *K. pneumoniae*.

PROCEDURE WITH ADDED STRESS FACTOR

Materials and Methods

A group of 100 White Leghorns, mixed sexes, 10 weeks old, were given, respectively, 1 cc. of standardized PPLO culture intraperitoneally three times at 48-hour intervals, and a dusting with a 500-dose vial of laryngotracheitis dust vaccine following the last injection and were kept under observation for 6 weeks.

A second group of similar birds was given, respectively, 1 cc. of standardized

Klebsiella culture intraperitoneally in the same manner as the first group, and also a dusting with larnygotracheitis virus dust vaccine. Observation was also for six weeks.

Birds were dusted above their heads in separated isolation pens with a vaccine gun.

Results

The birds receiving both the PPLO culture and laryngotracheitis virus showed mild respiratory symptoms six weeks after dusting. However, there was very little air-sac involvement, and the agglutination test for PPLO was weakly positive.

Birds receiving *Klebsiella* and laryngotracheitis vaccine showed nothing more than the usual lacrimation following dusting. Air sacs were clear, and PPLO antibodies were absent.

DISCUSSION

An earlier pilot trial had demonstrated that typical CRD lesions and respiratory symptoms—manifested by lacrimation, coughing, gasping, and diminished feed and water consumption, as seen in the field—could be attained by a series of intraperitoneal injections of a combined PPLO and porcine *K. pneumoniae* culture.

Inasmuch as previous work had indicated that stress factors appear to be essential for the clinical manifestation of the CRD syndrome, it was decided to expose birds through different avenues of infection with an organism that would aid the potential pathogenicity of the PPLO. As a result, an entity was chosen that was unrelated to the avian species and was utilized merely to enhance the PPLO from a synergistic standpoint or to create an added physiological stress on the part of the host, but not for the purpose of causing a double infection.

Of particular importance is the fact that, although it was possible to reproduce CRD experimentally by intraperitoneal injections, the same result was accomplished through a more natural route by aerosol inhalation. It is difficult to ascertain, however, whether the presence of the *Klebsiella* species actually served synergistically with the PPLO toward the over-all pathogenicity obtained, or whether the former organism created an added stress upon the defense mechanisms of the birds, thereby presenting a greater opportunity for the PPLO infection to become established. In this respect, then, it might be more appropriate to consider the *Klebsiella* as a potential stress factor rather than a synergistic entity, inasmuch as the data indicated little if any infectivity on the part of this organism to the host. With the uncertain status, at the present time, of the PPLO as a pathogenic entity, the factors involved in establishing its pathogenic role in CRD are of immediate importance.

The utilization of laryngotracheitis dust vaccine, as an apparent stress mechanism, did not duplicate the activity of the porcine culture. This result would indicate that a more severe type of stress, typified by the *Klebsiella* species, may be necessary for an experimental reproduction of the CRD syndrome. As a result, viral vaccines which can be used from time to time for tressing purposes, with the potential danger of infecting normal flocks nearby, need not be used for such purposes on the basis of the results obtained. The porcine *Klebsiella* species can serve as a stress mechanism with little danger of spreading a poultry infection since, apparently, the culture is nonpathogenic, per se, to chickens.

Comparatively, the intraperitoneal route of infection appeared to create a more severe form of the disease than the aerosol inhalation procedure. Whereas the former method elicited obvious respiratory symptomatology accompanied by thoracic and abdominal air-sac involvement and pericarditis, the latter did not demonstrate such outstanding symptoms, although these were discernible to a lesser degree. The results indicated that intraperitoneal njections rendered a generally acute septicemia, while aerosol inhalation terminated in a more localized and pneumonic infection.

SUMMARY

Several practical laboratory procedures can be utilized to reproduce CRD experimentally. Intraperitoneal injections of an avian PPLO culture and a porcine *K. pneumoniae* strain were successful in reproducing the CRD syndrome. A more natural route of infection, direct aerosol inhalation, using the same organisms, duplicated the former results, although the syndrome was not as pronounced as that which occurred with the intraperitoneal infection. The role of the *K. pneumoniae* appears to be that of a physiological stress factor on the host, which enhances the establishment of the PPLO infection. Simultaneously, the presence of the former may have a synergistic effect with the PPLO culture. Apparently, the use of laryngotracheitis vaccine, although a mild stress itself, is not as satisfactory as the *Klebsiella* species for such purposes.

The practical application of these experimental procedures can be utilized for future screening programs in the search for possible chemotherapeutic agents against the PPLO of CRD.

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INDUCED PPLO INFECTION IN YOUNG CHICKENS

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Introduction

Although pleuropneumonialike organisms (PPLO) are accepted as the causative agents of infectious turkey sinusitis, their role in chronic respiratory disease (CRD) of chickens has been more difficult to determine. Various investigators have reported that PPLO cause a mild disease or are nonpathogenic in the chicken.[1,2] The purpose of this study was to determine whether a reproducible system of PPLO infection of young chickens could be established for use in the study of therapeutic agents. Results of experiments involving 1300 chickens, in which inoculations of pure cultures of avian PPLO were used, will be reported.

It seemed that the best system of infection would be one that routinely would stimulate antibody titers, appreciably depress weight gains, produce typical gross and histological lesions, and permit recovery of the organism from a high percentage of the chickens. Previous experience indicated that the route of inoculation, the type of inoculum, and the strain of PPLO might be important. Based on these thoughts, the first five experiments were conducted to include as many variables as possible. The later experiments followed the early leads and gave additional information that substantiated the chosen system of infection.

Experimental Design

Broiler-type chickens, usually one day of age, were purchased from a commercial source and were maintained in isolation on our premises during an experiment.

For each experiment, chickens of the same age and from the same source were divided into numerically equal groups having the same average weight per chicken. The ages of the chickens ranged from 3 to 8 weeks in the 9 experiments conducted. Individual records were kept for each chicken. A number of antibiotic materials were examined as therapeutic agents and, in most instances, comparisons were made between groups of chickens which were (1) inoculated with PPLO and treated with an antibiotic; (2) inoculated and not treated; (3) not inoculated with PPLO, but treated with an antibiotic; or (4) neither inoculated nor treated. In this paper, only those birds that did not receive therapy will be considered.

The PPLO strains used were all obtained from other laboratories. TABLE 1 lists the pertinent information concerning each strain. Strains 1051, 1058 and 3720 were isolated from chickens that had been inoculated with a particular strain of PPLO, but no attempt was made to identify the reisolated strains to be the same as the original strain. Grumbles' phenol-red broth[3] without inhibitors or 7-day-old embryonating chicken eggs were used for propagation of the PPLO. All strains possessed colony and cell morphology typical of PPLO, and they fermented maltose within 24 hours.

Broth cultures for inoculation of chickens were prepared by incubation of the ulture at 37° C. for from 24 to 30 hours, by centrifugation at 2500 rpm for 30 iin. in a Servall Model G angle-head centrifuge, and by resuspension of the ediment in one half of the supernatant.

Embryonating hen's-egg inocula were prepared by injecting the PPLO strain nto the yolk sac of 7-day-old embryonating eggs. After incubation for 5 ays at 37° C. the eggs were chilled overnight and the yolk-sac membrane and ontents aseptically removed. These materials were diluted to 10 times volme with yeast-hydrolyzate phenol-red broth and mixed for 3 min. in a Waring Blendor. The suspension was centrifuged at 1000 rpm for 30 min. and the upernatant used as inoculum within 24 hours after harvest.

Titrations of either inoculum were conducted using tenfold serial dilutions in henol-red broth and plating one-tenth ml. of each dilution on plates of PPLO gar (Difco) containing 1.5 per cent PPLO serum fraction (Difco). Plates nd tubes were incubated at 37° C. and examined for growth on the fourth day. Titers of the inocula in the various experiments ranged from 10^3 to 10^6 PPLO er ml.

TABLE 1

PPLO STRAINS USED IN THESE STUDIES

Our number	Original number	Source	Furnished by
699	P12027	Chicken	F. H. White
295	S6	Chicken	H. E. Adler
1989	Texas C1	Chicken	L. C. Grumbles
1990	Texas C2	Chicken	L. C. Grumbles
1991	Texas T1	Turkey	L. C. Grumbles
1051, 1058	—	Reisolated at 2 weeks postinjection from chickens inoculated with 295	
3720	—	Reisolated at 4 weeks postinjection from a chicken inoculated with 699	

After collection of blood specimens and tracheal swabs, chickens were inoculated by way of either a single or a combination of 2 simultaneous routes. These included intravenous (IV), intratracheal (IT), intrasinus (IS), intra-air sac (AS), or aerosol (A). Five-tenths ml. of inoculum was used in the IT and IS routes. Two-tenths ml. of inoculum was used in each of the other routes except aerosol, whether in a single or combined administration. For aerosol, a group of 5 chickens was placed in a closed plastic sack of approximately 1 cubic foot volume and atomized with 5 ml. of broth culture (Rexall model R 1541 Defender Atomizer). Contact was maintained for approximately 1 hour. Small air holes were punched in the sack to prevent asphyxiation.

Serologic techniques included both the serum-plate (SP) agglutination test using antigen prepared from PPLO strain 295 following the method of Adler and Yamamoto[4] and the hemagglutination inhibition (HI) test using commercial antigen.* Sera from chickens were tested to detect the presence of antibodies before inoculation and at various times following inoculation.

All of the chickens in an experiment were weighed individually at weekly intervals beginning at the time of inoculation.

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* Antigen obtained from Connaught Medical Research Laboratories, Toronto, Canada.

Once a week 5 chickens from each group were sacrificed and examined for gross pathological changes. Air-sac lesions were graded numerically according to severity. No visible change of the membranes was recorded as number 1, and thickened, caseous membranes were classed as number 4, with other gradations between them.

Specimens were collected at necropsy from the trachea, lungs, and heart for histological study. In the earlier experiments, tissues were collected from only a few chickens in each group. Confirming the work of Jungherr *et al.*,[5] it became evident that histological examination was valuable for interpretation, and specimens were collected from one half of the birds in each group.

Isolation of PPLO was attempted both before and after inoculation of the infectious material. Specimens collected from exudate or tissue, or swabs from air-sac lesions or tracheae were placed in tubes of a modified Hofstad and Doerr's chicken-infusion medium.[6] The medium was modified by the addition of 0.5 per cent dextrose and 0.025 per cent phenol red, and by the substitution of 1.5 per cent PPLO serum fraction (Difco) for 20 per cent chicken serum. Thallium acetate (1:2000) and penicillin (1000 U./ml.) were added as bacterial inhibitors. Cultures were transferred 4 times at 3-day intervals in liquid medium with plating of each passage on PPLO agar containing serum fraction. Cultures were deemed positive when morphologically typical PPLO colonies were found on the agar.

At least one half of the air-sac lesions in any group of chickens was cultured for the presence of bacteria other than PPLO. A sterile swab was rubbed over the lesion and then smeared on the surface of both an eosin-methylene-blue agar plate and a 5 per cent horse-blood agar plate. Observations for growth were made 24 hours later, and the organisms were then classified by colony and cell morphology and by Gram-stain reaction.

In most of the experiments, 3 chickens were selected at random from each group at the end of the experiment, and an attempt made to isolate the viruses of Newcastle disease and infectious bronchitis from lung and tracheal tissues of each. The specimens were carried for 3 blind passages in embryonating hen's eggs and were discarded if no changes were observed in the embryos.

Results and Discussion

Antibody response. In experiments 1 and 2 (TABLES 2 and 3) the results of the SP and HI tests on sera from the same chickens were found to correlate in about 93 per cent of the cases; therefore, in later experiments only the SP test was used. All of the PPLO strains tested, except strains 3720 and 1990, stimulated measurable blood titers by all routes in about 80 per cent of the chickens tested by 2 weeks postinoculation. With IV-AS inoculation of strain 699, almost all of the remainder of the chickens were positive by 4 weeks. Strain 1990 produced a much lower number of reactor chickens when injected by the IV route, but compared favorably with the other infective strains in the other routes. The type of inoculum did not appear to influence the stimulation of antibody titers, since similar results were observed with all strains of PPLO after administration of either inoculum.

Strain 3720 (experiment 5) did not stimulate an antibody response in any

TABLE 2

FECT OF PPLO STRAIN, INOCULUM, AND ROUTE OF ADMINISTRATION ON YOUNG CHICKENS: EXPERIMENT 1

PPLO strain	Inoculum and route*	Average weight gain (gm.)†	Gross air-sac lesions‡	Histological lesions‡	Cultural isolation of PPLO from tracheal swabs	
					Time postinoculation	
					2 weeks	4 weeks
699	B, IV	485	5/15§	2/2§	10/15§	7/8§
	B, IT-IS	585	7/15	1/2	15/15	7/8
	E, IV	610	0/15	1/2	3/15	8/8
	E, IT-IS	570	8/15	1/1	15/15	6/8
295	B, IV	405	3/15	0/2	3/15	1/8
	B, IT-IS	650	11/15	0/2	14/15	6/7
	E, IV	450	2/15	0/2	3/15	8/8
	E, IT-IS	600	8/15	0/2	14/15	4/8
oninoculated		630	0/15	1/3	0/15	1/8

* Key: B, broth; E, egg yolk; IV, intravenous, IT-IS, combined intratracheal-intrasinus utes.

† Fifteen chickens in each group with an initial average weight of 1260 gm./chicken; oculated at 8 weeks of age and observed for 5 weeks.

‡ Necropsy was performed at 4 and 5 weeks postinoculation.

§ Number positive/number examined.

TABLE 3

FFECT OF PPLO STRAIN, INOCULUM, AND ROUTE OF ADMINISTRATION ON YOUNG CHICKENS: EXPERIMENT 2

PPLO strain	Inoculum and route*	Average weight gain (gm.)†	Gross air-sac lesions‡	Histological lesions‡	Cultural isolation of PPLO from tracheal swabs	
					Time postinoculation	
					2 weeks	4 weeks
699	B, IV	415	12/15§	1/2§	11/15§	5/10§
	B, IT-IS	680	6/14	1/2	10/15	6/10
	E, IV	536	8/13	2/2	8/14	7/8
	E, IT-IS	615	9/15	2/2	13/14	6/10
1051	B, IV	525	9/13	1/2	13/15	7/9
	B, IT-IS	635	2/14	1/2	11/15	7/9
1058	E, IV	475	8/15	0/2	10/15	8/10
	E, IT-IS	575	10/15	2/2	12/15	7/10
oninoculated		802	0/14	1/2	0/15	0/10

* Key: B, broth; E, egg yolk; IV, intravenous; IT-IS, combined intratracheal-intrasinus utes.

† Fifteen chickens in each group with an initial average weight of 410 gm./chicken; oculated at 6 weeks of age and observed for 6 weeks.

‡ Necropsy was performed at 4 and 6 weeks postinoculation.

§ Number positive/number examined.

of the inoculated chickens by any route as measured with PPLO strain 29 antigen. Since the other measurement methods also indicated that this strai had not produced appreciable changes by 2 weeks postinoculation, the experiment was terminated at that time. The detailed data for experiment 5 wil not be presented in this paper.

Serology was useful to determine if all chickens of an inoculated group wer infected and if uninoculated groups remained uninfected during an experiment

Suppression of weight gains. In most instances the pathogenic strains o PPLO in these studies caused appreciable depression of weight gains in inoculated chickens as compared with their controls.

It was apparent in almost every experiment that the IV inoculation led t the greatest depression of weight gains. TABLES 2, 3, and 4 show this wit both strains 699 and 295, and it is shown in TABLE 5 with strains 1989 and 1991 Weight suppression by other routes was not as marked, but usually the inoculated chickens showed lower average weights than the uninoculated. Strai 1990, as illustrated in TABLE 5, had relatively less effect by all routes than ha any other strain. In most instances, the type of inoculum did not influenc greatly the amount of weight depression.

TABLE 4

EFFECT OF BROTH-CULTURE PPLO STRAIN 699 ON YOUNG CHICKENS WHEN INOCULATED B VARIOUS ROUTES: EXPERIMENT 3

Route of inoculation*	Average weight gain (gm.)**	Gross air-sac lesions†	Histological lesions†	Cultural isolation of PPL from tracheal swabs	
				Time postinoculation	
				2 weeks	4 weeks
Intravenous	822	0/9‡	0/5‡§	7/9‡	0/9‡
Air sac	1002	2/10	0/5	4/9	0/9
Aerosol	1047	6/10	0/5	7/9	2/9
Noninoculated	1172	0/10	0/5	0/9	0/9

* Ten chickens in each group; inoculated at 4 weeks of age and observed for 4 weeks.
** Average initial weight of 478 gm./chicken.
† Necropsy was performed at 6 weeks postinoculation.
‡ Number positive/number examined.
§ Specimens of trachea were not examined.

Strain 699, administered by the IV-AS route, most consistently produce suppression of weight gains. The information in TABLE 6 shows that it suppressed average gains 66 to 85 per cent.

The largest differences in weight gains between inoculated and noninoculate chickens were measured during a period of rapid growth. With broiler-typ birds, this was found to be between 3 and 10 weeks of age.

Because a system was desired that would show large differences, the weigh gain data were not subjected to statistical analyses. A difference of 100 g between the average weights of any 2 groups was chosen arbitrarily as th smallest difference to be considered meaningful.

Incidence of air-sac lesions. The strains of PPLO used in these experimen produced gross air-sac lesions that corresponded relatively to suppression

TABLE 5

FECT OF BROTH-CULTURED PPLO STRAINS ADMINISTERED BY VARIOUS ROUTES ON YOUNG CHICKENS: EXPERIMENT 4

PLO strain	Route of inoculation*	Average weight gain (gm.)†	Gross air-sac lesions‡	Histological lesions	Cultural isolation of PPLO from tracheal swabs	
					Time postinoculation	
					2 weeks	4 weeks
1989	IV	697	0/9§	0/3§	2/9§	8/9§
	AS	734	7/9	0/3	1/8	9/9
	A	746	4/9	0/3	2/9	8/9
1990	IV	802	1/9	0/3	0/9	8/9
	AS	788	5/9	1/3	1/9	8/9
	A	796	0/9	0/3	2/9	9/9
1991	IV	599	1/9	1/3	3/9	6/9
	AS	710	7/9	3/3	2/9	3/9
	A	693	7/9	2/3	4/9	2/9
ninoculated		873	0/9	0/9	0/9	0/9

* Nine chickens in each group inoculated at 4 weeks of age and observed for 4 weeks post- oculation.

† Initial average weight 328 gm./chicken.

‡ Necropsy was performed at 4 weeks postinoculation.

§ Number positive/number examined.

TABLE 6

FECT ON YOUNG CHICKENS OF BROTH-CULTURED PPLO STRAIN 699 INOCULATED BY THE COMBINED INTRAVENOUS-AIR SAC ROUTES AS COMPARED TO NONINOCULATED CHICKENS: EXPERIMENTS 6, 7, 8 AND 9

xperi- ment umber	Group*	Beginning average weight (gm.)	Average weight gain (gm.)	Gross air-sac lesions†	Histological lesions	Cultural isolation of PPLO from the trachea	
						Time postinoculation	
						2 weeks	4 weeks
6	Inoculated	341	452	22/24‡	11/18‡	2/4‡	19/25‡
	Noninoculated	341	856	0/25	3/21	0/5	0/24
7	Inoculated	374	554	22/25	18/18	4/5	3/24
	Noninoculated	374	717	0/25	1/20	0/5	0/25
8	Inoculated	360	656	24/25	19/20	5/5	2/12
	Noninoculated	360	846	1/25	5/21§	1/5	0/11
9	Inoculated	319	575	24/24	15/15	3/5	0/12
	Noninoculated	319	736	0/25	0/15	0/5	0/13

* Forty chickens per group, inoculated at 3 or 4 weeks of age and observed for 4 weeks. ecropsy was performed on 15 chickens during the experiment, and 25 chickens were carried rough to the end of the experiment.

† Necropsy was performed on all chickens reported at 4 weeks postinoculation.

‡ Number positive/number examined.

§ All lesions found at 4 weeks postinoculation.

weight gains. A direct AS inoculation offered better opportunity for air-sa infection even though some strains produced air-sac lesions by other routes Much variation occurred in the incidence of air-sac lesions in chickens inoculated by the IV and the IT-IS routes, as shown in TABLES 2, 3, and 5. Th aerosol route seemed to result in a fair number of air-sac lesions, but for ou use the system was considered too cumbersome and too difficult to control.

TABLE 5 shows that the direct air-sac route of inoculation produced a hig incidence of air-sac lesions with strains 1989, 1990, and 1991. However, the IV route produced the greatest amount of weight suppression. It was reasone that a combined IV-AS route would result in the most consistent reproductio of weight suppression and air-sac lesions. TABLE 6 confirms this reasonin with results observed in 4 experiments with the IV-AS inoculation.

The low incidence of air-sac lesions following AS inoculation of strain 699 as indicated in TABLE 4, cannot be explained. This was a singular instanc with this route of inoculation.

The gross air-sac lesions following IV-AS inoculation of strain 699 ofte extended from the injection site to the adjoining air sacs, usually within 3 t 4 weeks. Typical lesions were seen commonly and ranged through all degree of severity.

In extreme cases, large pieces of dehydrating caseous exudate were foun lying adjacent to thickened air-sac membranes. Pericarditis and perihepati fibrin deposition were seen in a very few chickens in all of the experiments.

Incidence of histological lesions. The occurrence of histological lesions i chickens inoculated with pathogenic strains was contrasted with the almos complete absence of lesions in the uninoculated controls. In all experiment histological changes in the tracheae of inoculated chickens far outnumbered th changes in the other tissues examined. The IV-AS route of inoculation wit PPLO strain 699 appeared to induce the various histological lesions most consistently.

Tracheal changes included congestion, thickening, and lymphoid or plasm cell infiltration of the mucosa, together with epithelial hyperplasia, or metaplasia, and a loss of epithelial cells, glands, and cilia. All combinations an degrees of these changes were seen.

In some instances lymphocytic infiltration of the lung interstitium or pu cells in the alveoli were seen. In a few chickens, pericarditis with lymphocyti infiltration in the adjacent myocardium was noted, but more often there was lymphocytic infiltration of the myocardium without necrosis. Unfortunately specimens of air sacs were not included in histological studies.

Comparison of the changes in the selected tissues at different times indicate gradually progressive lesions that did not show signs of healing by 4 week postinoculation.

It was found that the use of numerical grading of the gross air-sac lesion was very helpful in comparisons between groups of inoculated chickens. similar indexing system of histological changes might be even more definitiv in these comparisons.

Cultural isolation of PPLO. Isolation of PPLO by culture from inoculate chickens was of value in demonstrating the establishment of infection. However, the fastidious nature of the PPLO and the interference of contaminatin cteria were two complicating factors that required consideration. It seemed at the culture results were of greatest significance when correlated with other easurement methods.

In most experiments using strain 699, the incidence of recovery of the or-nisms at 2 weeks postinoculation was high. This was not always true at 4 eeks postinoculation, as seen in TABLES 2, 3, and 6. In TABLE 5 the incidence recovery of PPLO was much higher at 4 weeks than at 2 weeks postinocula-on. It should be noted here that the high rate of reisolation did not cor-spond with other pathological changes produced by strain 1990.

Other findings. All attempts to isolate the viruses of Newcastle disease and fectious bronchitis were negative in these experiments. When air-sac lesions ere cultured for the presence of secondary bacteria, the agar plates were sually negative for any growth. The bacterial colonies observed were identi-ed as either nonhemolytic Gram-positive cocci or as having the colony char-teristics of the *coli-aerogenes* group.

In the system using PPLO strain 699 by the IV-AS route, readily apparent les and sneezing usually were observed in inoculated chickens. The signs egan between the second and third weeks postinoculation and continued rough the fourth week, when the experiments were terminated.

Conclusions

The chicken infection system that has been chosen for evaluation of thera-eutic agents uses the IV-AS inoculation of a broth-propagated PPLO strain. fter preliminary blood tests and tracheal cultures to determine the status of e chickens, they are inoculated at 3 weeks of age. Observations for the fect of infection and treatment are continued through a 4-week period. Forty ickens are included in each group. Of these, 25 of equal average initial eight are weighed at weekly intervals. Fifteen others in each group are not eighed, but necropsies are performed on groups of 5 at weekly intervals. All iteria of measurement are used as previously described in this paper. Al-ough this appears to be a severe infection system, it has been demonstrated at certain therapeutic agents will alter the course of the infection as meas-red by these criteria.

Confirmation of the identity of the etiological agent in these studies was ccomplished by isolation in culture of PPLO and antibody response. The ilure to demonstrate the viruses of Newcastle disease and infectious bronchitis nd the low incidence of bacterial isolations supported the role of the PPLO as e infectious organism.

The possible loss of pathogenicity of strain 699 appears to have been avoided these experiments by using Grumbles' medium or low-numbered serial assages in embryonating eggs. This strain had been passed 27 times in rumbles' medium and once in embryonating eggs before being sent to our boratory. Passages used in our experiments were between numbers 5 and 4. The continued virulence of this strain in these experiments indicates that pproximately 30 to 40 passages in Grumbles' medium did not cause loss of athogenicity. The other strains were not used in a sufficient number of ex-eriments to allow observation of any changes that might have occurred.

If a PPLO system is to be interpreted adequately, it is important that all these measurement criteria be used rather than just one or two. Results ob tained with some of the methods of measurement do not always parallel eac other closely, but add to the over-all interpretation of the infection.

It was recognized that a problem was introduced by the fact that the chicken in these studies were obtained from commercial sources and probably were no from PPLO-free stock. In a few instances positive blood titers were found o isolations of PPLO were made from non-inoculated chickens during an exper ment, and this may have indicated a natural infection. Because of the larg differences found between the inoculated and the control groups, the youn age of the chickens at the time of inoculation, and the large inoculum give to each chicken, it was felt that the problem was not significant.

Summary

This paper describes the results of nine experiments on a system for infectio of young chickens with avian PPLO and the methods of measurement of thi infection. The measurement regime included serology, weight gains, recover of PPLO by culture, the incidence of gross air-sac lesions, and the incidence o histological lesions in selected tissues. The evidence indicated that all of thes methods were necessary to study the infection adequately.

These data show that a pathogenic avian strain of PPLO propagated i Grumbles' broth and inoculated by the combined intravenous-intra-air sa routes consistently produced a measurable infection in young chickens.

Acknowledgment

We gratefully acknowledge the technical assistance of M. G. Richmond, M Moore, G. Brinkman, J. Shoufler, and G. Pedigo in conducting the experi ments; of P. N. Harris in the histological studies; and the help of C. Leber Mildred Haines, and Shirley Johns in preparation of the manuscript.

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PATHOLOGICAL AND IMMUNOLOGICAL OBSERVATIONS CONCERNING AVIAN PLEUROPNEUMONIALIKE ORGANISMS*

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Chronic respiratory disease (CRD) in chickens and infectious sinusitis (IS) turkeys have a common etiological agent that has been identified in recent ears as one of the pleuropneumonialike organisms (PPLO).[1]

Delaplane and Stuart[2] first described CRD in chickens as characterized by isal discharge, respiratory rales, slow rate of spread, persistency of the clinical gns, moderate decline in egg production, and loss in body weight. Natural itbreaks were observed generally in laying flocks. The disease was repro- iced in young chicks and mature birds when inoculated either with the em- yo-propagated agent or with material from naturally infected chickens.

Infectious sinusitis in turkeys has been recognized as a distinct disease entity r more than half a century.[3,4] The disease is characterized by shaking of ie head, nasal discharge, frothy lacrimation, distention of the infraorbital nuses with mucogelatinous exudate, dyspnea, coughing, pneumonia, and in- ammation of the air sacs. Delaplane[5] and other investigators[1,6-10] have em- iasized the fact that CRD and IS are caused by the same etiological agent.

Losses from these diseases among chicken and turkey flocks may be very ostly to the producer, especially to the broiler grower. Frequently CRD is omplicated with other diseases that aggravate the severity of the infection id increase the mortality.[11,12] The prevalence and economic importance of RD have been reported in several countries.[13]

This paper comments briefly on the pathological and immunological aspects CRD and is based on observations of experimental and natural cases of the isease.[6,12,14]

Pathological Aspects

The gross pathological observations of the natural disease in chickens and irkeys reveal an involvement of the upper and lower respiratory tract, includ- ig the air sacs. The gross changes in the various tissues have occurred in ie following decreasing order of frequency: trachea, air sacs, sinuses, and lungs. he mucosa of the infraorbital sinus may reveal edema, congestion, and pete- iiae, and the sinus cavity may contain clear or turbid cohesive mucus. Fre- uently accumulation of the mucus within the sinuses of turkeys may cause a iarked distention of the wall, while in chickens this is less conspicuous (FIGURES , 2*a*, *b*, and *c*). Complicating bacterial infections in the sinus frequently iay cause a purulent exudate. The microscopic pathology reveals that the iucosal thickening is due largely to subepithelial infiltration of lymphocytes

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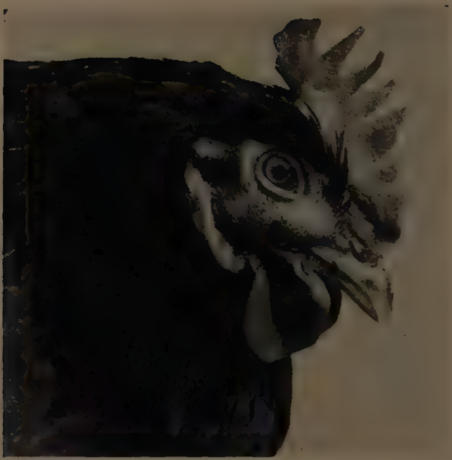

FIGURE 1. Hen inoculated by the intrasinus and intratracheal routes with allantoamnioti fluids containing the CRD agent. Distention of the right sinus, nasal discharge, and trachea rales were evident 7 days postinoculation, when the photograph was taken.

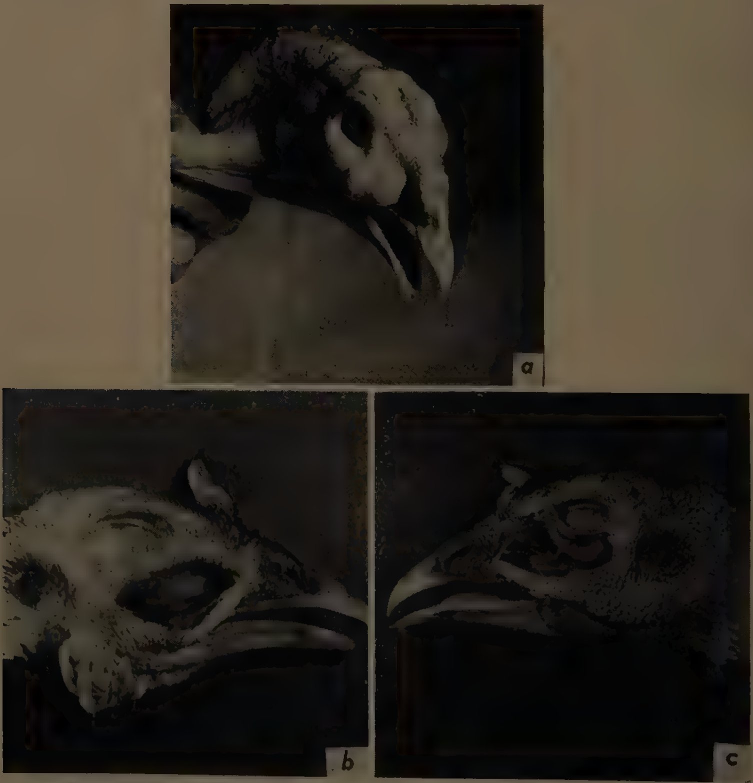

FIGURE 2. Mature male turkey, MX 29686, photographed 22 days after the right sinu had been inoculated with nasal exudate from a field case. (*a*) Distention of inoculated righ sinus and nasal discharge. (*b*) Right sinus, with lateral wall incised and edges spread apar showing thickened grayish discoloration of the mucosa. (*c*) Normal left sinus, with latera wall incised and edges spread apart.

l large mononuclear phagocytes (FIGURE 3). Lymphoid foci are conspicuous he mucosa which may be detected on the surface as raised lymphoid follicles GURE 4). Slight to diffuse hemorrhage is found in many cases, and the thelium reveals a hyperplasia, especially of the mucous glands. The sinus date contains mucocellular elements and epithelial fragments.

The gross pathology of the trachea is similar to that observed in the sinuses, sal passages, and larynx. Frequently the upper trachea is involved more ensively than the lower portion. In early stages of the disease the tracheal

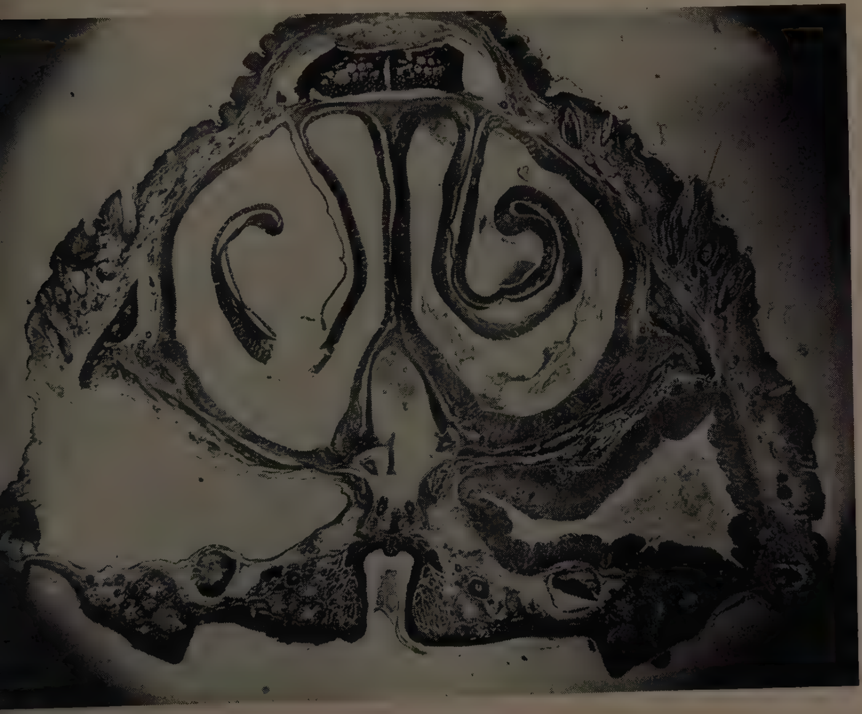

FIGURE 3. Transverse section through nasal passages and infraorbital sinuses of a 16-week-d experimental chicken. Marked mucosal thickening in the right sinus and nasal passage ter inoculation. ×6.

ucosa may reveal small raised follicles that present a roughened "beaded" ppearance on the surface. However, in cases that exhibited signs of tracheal ales, mucosa reveals edema and congestion, and is coated with turbid viscid ucus. Microscopically, the tracheal mucosa reveals a mononuclear infiltraion, lymphofollicular reaction, and epithelial hyperplasia, and is coated with ucocellular exudate (FIGURE 5). The lymphoid foci in the mucosa often ontain small hemorrhages. The epithelial changes are characterized by loss f cilia, hydropic degeneration, and elongation of the mucous glands.

In the lung the gross findings are usually slight, but severe involvement sually is observed when the disease is complicated with other infections. Frequently exudate is observed in the primary bronchus accompanied by a thick ening of the bronchial mucosa. Necrotic caseous cores are frequently observe in the bronchial lumina and grayish pneumonic areas occurring along th dorsolateral posterior border of the lung. These areas may extend deeply int the lung. Microscopically, an exudative and proliferative response is note with fibrin and heterophils occurring in the secondary and tertiary bronch contiguous to the primary bronchi (FIGURE 6). Cylindrical granulomas wit necrotic cellular cores ringed by multinucleated giant cells are observed ofte in the natural disease (FIGURE 7). Focal lymphocytic proliferation is commo in the tertiary bronchi and interstitial tissue for both the natural and experimental disease.

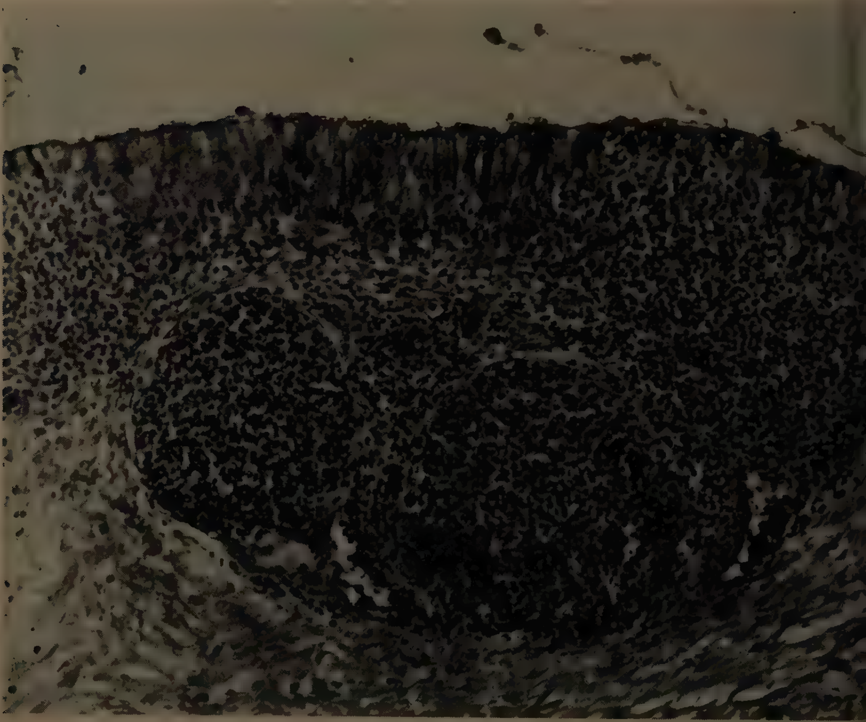

FIGURE 4. Transverse section of right sinus in a 7½-month-old chicken, showing 2 lymphoid foci and infiltration of inflammatory cells into the hyperplastic epithelium. ×170.

In the air sacs the gross pathology is usually very extensive and striking While all the air sacs may be affected, the lesions frequently are confined t the posterior thoracic and abdominal air sacs. In the early stages the air-sa membranes may reveal a slightly turbid and edematous appearance that ma be followed by an increased vascularity. This is followed by an exudatio coating the membrane with whitish-yellow exudate. In some instances mphofollicular reaction presenting a "beading" effect of the membrane may observed (FIGURE 8).

As the inflammatory changes progress, the membranes increase in thickness d may be slightly to heavily coated with yellowish exudate. In severe cases e membranes are markedly thickened, and the air-sac cavities may contain pious amounts of caseous exudate. Consequently, meat flocks with severe RD infection suffer a high rate of carcass condemnation when submitted for aughter. The microscopic pathology of the air sacs consists of focal prolifera-

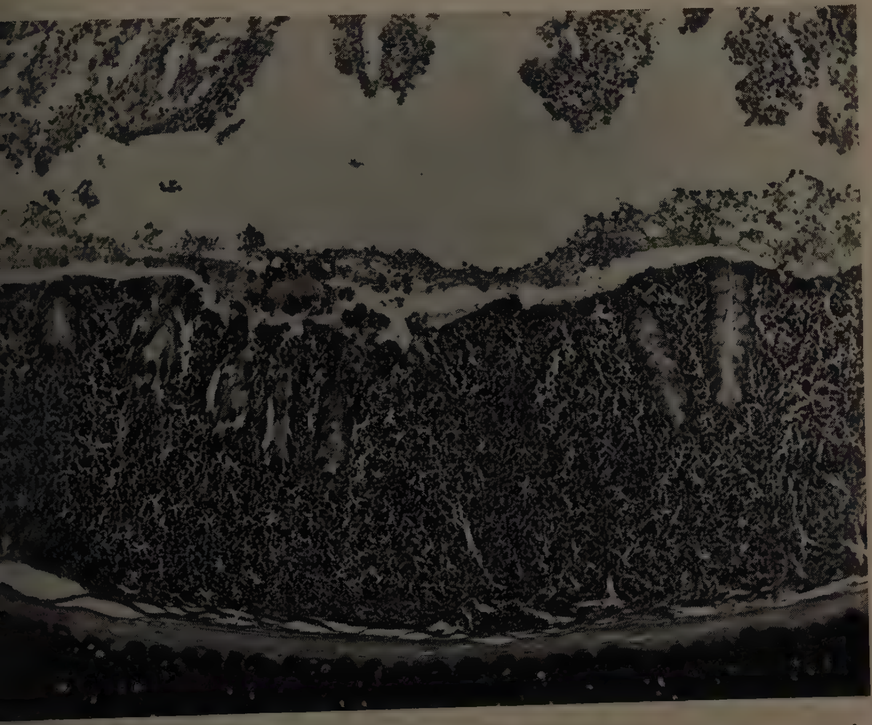

FIGURE 5. Trachea of an 18-week-old chicken, showing infiltration of the lamina propria y lymphocytes and mononuclear phagocytes, marked elongation of the mucous glands, and nucocellular exudate in the lumen. ×100.

tion of lymphocytes and an exudation consisting of fibrin, mononuclear cells, ymphocytes, and heterophils (FIGURES 9 and 10). Evidence of fibroblast invasion of the exudate is noted in chronically affected membranes.

Since CRD appears to be primarily an egg-borne disease at this time, a brief lescription of the lesions in the embryo and newly hatched chick may be indicated. The gross findings in embryos consist of tracheal, lung, and air-sac involvement. Complete occlusion of the lumen of the trachea with necrotic cellular debris and perivascular infiltration with inflammatory cells, septal edema, and cellular exudation of the lungs have been observed. Necrotic cellular plugs and granulomas are somewhat similar to the lesions seen in experimental chickens. The air sacs may reveal a thickening of the membranes and marked exudation in the lumen. These embryo lesions are considere pathognostic for CRD at the present time.

Immunological Aspects

Delaplane and Stuart[2] reported that chicks recovered from CRD appear t be refractory to the disease upon re-exposure. Field observations also sugges that, once a flock has experienced an outbreak of the disease and recovere from the clinical signs of the infection, it will not manifest a second outbrea of the malady. However, the authors have observed that both turkeys an chickens, having had previous contact with the infection, may be only partiall refractory to the agent upon re-exposure.

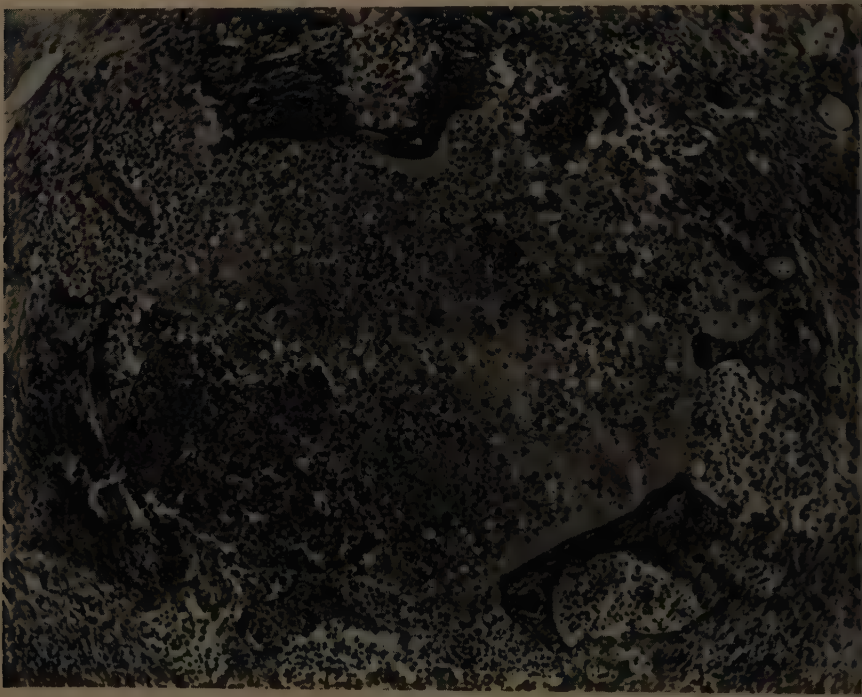

FIGURE 6. Lung of a 2½-month-old chicken, showing extensive fibrinocellular exudat and necrotic debris in the lumen of a tertiary bronchus. Exudative pneumonia. ×95.

Jungherr *et al.*[15] were the first to devise a serologic test for CRD that demon strated the PPLO to be antigenic and to be agglutinable by its specific immun serum. Later other investigators[7,10,16] demonstrated that different serologi methods could be employed to detect antibodies for the PPLO and also tha certain serotypes existed. These serologic techniques have given great impet to the approaches to a better understanding of the disease.

Chickens 4 weeks and older, when exposed to the CRD agent propagated i artificial medium, embryonating eggs, or in birds, may develop agglutinins f PPLO from 7 to 21 days postinoculation. The California S-6 strain of PPL s used in the serum-plate agglutination test.[10] TABLE 1 shows that among chicks approximately 6 weeks of age the sera of 17 chicks definitely agglutited PPLO antigen 14 days after exposure to the agent. All of these chicks vealed marked typical CRD lesions in the tracheas and air sacs when sacri-

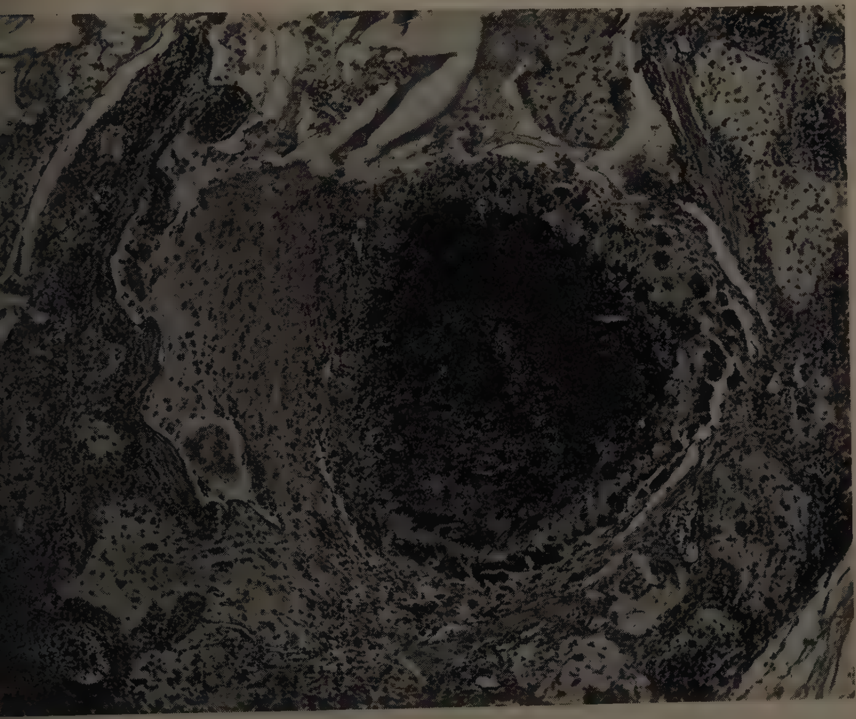

FIGURE 7. Lung of a mature chicken with a granuloma in the lumen of a tertiary bronchus iowing a necrotic core surrounded by multinucleated giant cells. ×85.

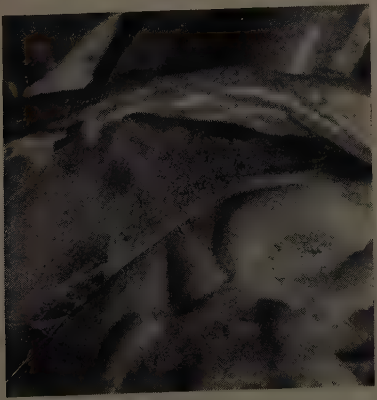

FIGURE 8. Lymphofollicular or "beading" reaction in the left lesser abdominal air sac of nature female turkey, MX 29688, 24 days after intratracheal inoculation with nasal exudate rom a field case.

ficed 25 days postinoculation. There appeared to be some correlation between the appearance of respiratory signs and the appearance of agglutinins.

In a second experiment that involved 2 groups of birds, each group being subdivided into 3 subgroups, agglutinin response was observed in 49 of 50 inoculated birds 14 days postinoculation. In a group of 22 noninoculated birds placed in contact with the inoculated birds at the start of the experiment, agglutinins were detected in the sera of 2 birds on the twenty-first day, and in 18 birds on the thirty-fifth day, and in all of the 22 birds by the fifty-fifth day. In a subgroup of 22 noninoculated brids placed in contact with the previous 2 groups 14 days after the start of the experiment, the agglutinin response was similar to that of the other contact group. A second group, also composed of 3 subgroups, was infected in the same manner as the first group, but was also medicated with chlortetracycline (200 gm. per ton of feed). The medication was started 2 weeks after the experiment was initiated. It may be noted from the result that the agglutinin response was delayed and that not all birds developed antibodies (TABLE 2). The clinical signs and lesions of the disease in the second group were not as pronounced as in the first group.

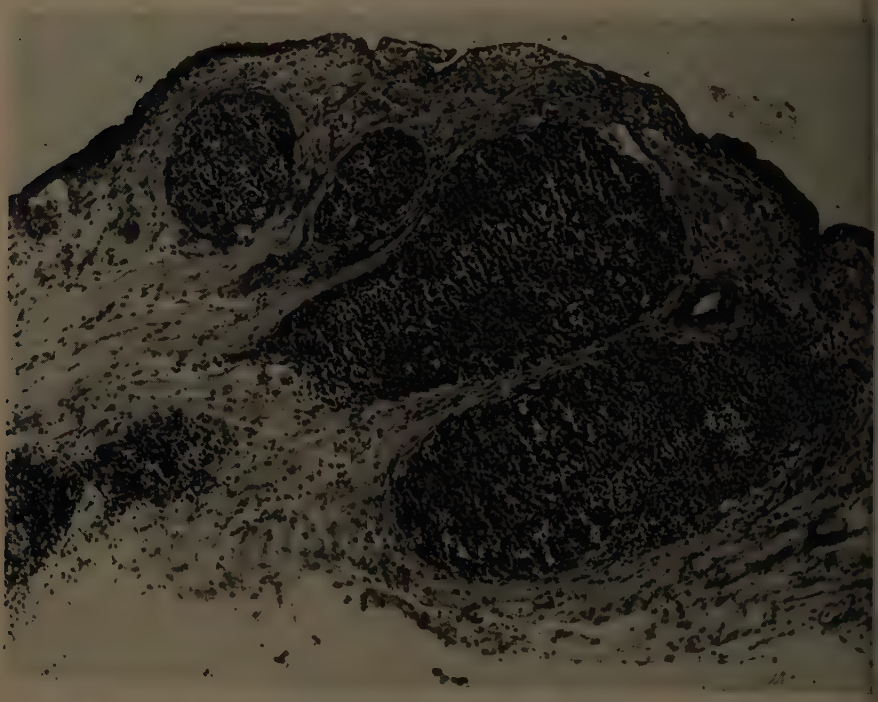

FIGURE 9. Air sac of a 7-week-old experimental chicken, showing lymphofollicular reaction. ×100.

Contact transmission of CRD between naturally infected and susceptible stock may be measured by agglutinin production. Susceptible stock, 50 hens and 8 males placed in contact with 50 serologically positive hens, revealed the resence of agglutinins approximately 30 days after initial contact (TABLE 3). fter 3 months' cohabitation essentially all the birds were positive. It is of nterest to note that transmission of the infection occurred with only slight linical evidence of the disease in a few birds. Similar results were obtained ith another group of birds, with the rate of spread somewhat slower (TABLE). The delayed appearance of agglutinins suggests that widespread transnission in a flock may be related to the development of active cases of the dis-

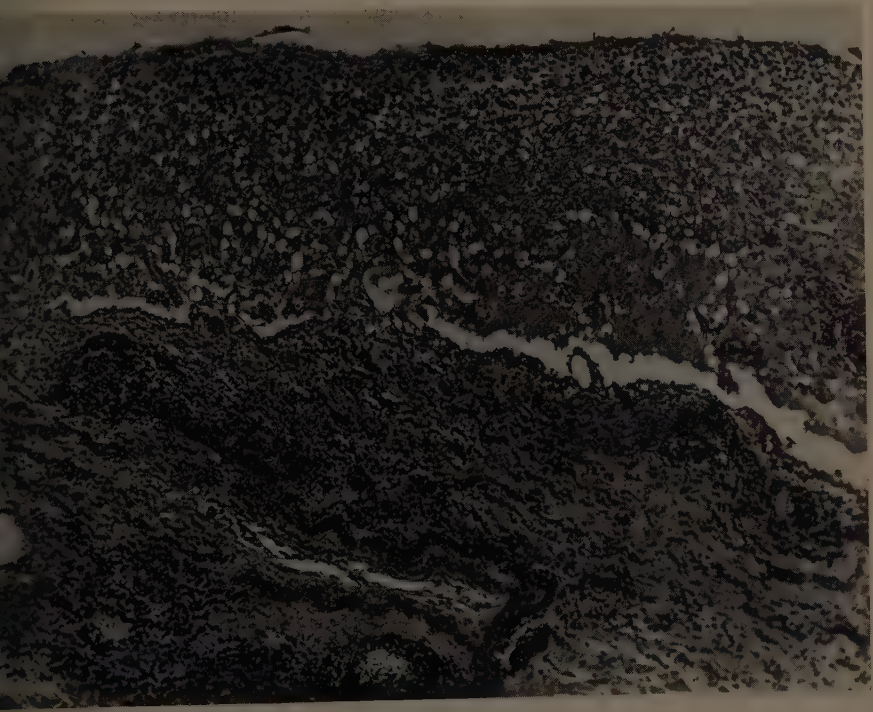

FIGURE 10. Air sac of a 5-week-old experimental chicken. Marked cellular exudate adherent to air-sac membrane, showing diffuse proliferation of lymphocytes and mononuclear phagocytes. ×85.

TABLE 1
AGGLUTININ RESPONSE IN 19 CHICKENS INOCULATED WITH CRD AGENT

Days tested postexposure	Chickens classified by degree of reaction				
	0	1	2	3	4
0	19				
7	14	5			
14	0	2	7	10	
19	0	0	0	2	17
25	0	0	0	1	18

Age, 6½ weeks; breed, White Plymouth Rocks; inoculum, 595-4 embryo material; route, right sinus and trachea; dose, 0.5 ml.

ease among the birds. It should be noted that the degree of reaction in th naturally infected hens was very similar in the different tests during the months. The above results substantiate findings by other investigators tha the CRD agent can stimulate the production in fowl of agglutinins that wil react with the PPLO. The mode of exposure to the CRD agent may influenc the rate of agglutinin response.

The rapid-serum-plate agglutination test also has been employed to deter

TABLE 2

AGGLUTININ RESPONSE IN CHICKENS EXPOSED TO THE CRD AGENT

Days tested postexposure	Number of birds positive in the various groups					
	Nontreated group			Treated group		
	Inoculated (50 birds)	Contacts I* (22 birds)	Contacts II† (20 birds)	Inoculated (50 birds)	Contacts I* (22 birds)	Contacts II (22 birds)
0	0	0	0	0	0	0
8	23	0	0	6	0	0
14	49	0	0	40‡	0	0
21	50	2	8	45‡	0‡	2
28	50	4	19	45	0	8
35	50	18	20	46	6	10
42	50	20	20	46	11	12
49	50	21		45§	12	
55	49‡	22		47	14	

* Birds placed in contact with inoculated birds at time of CRD exposure.
† Birds placed in contact with inoculated birds 2 weeks post CRD exposure.
‡ One bird dead.
§ One bird not tested.
Age, 9½ weeks; breed, White Leghorn males; inoculum, BVH-10; route, trachea; dose 0.5 ml.

TABLE 3

CONTACT TRANSMISSION OF CRD AMONG CHICKENS AS DETERMINED BY THE AGGLUTINATION TEST

Date of test	Positive	Negative contact		
	Group I	Group II		
	50 BR hens No. positive	53 WL hens No. positive	4 RIR males No. positive	4 WR males No. positive
9/30/58	50	0	0	0
10/23/58	50	0	0	0
10/30/58	50	1	2	2
11/6/58	50	3	4	3
11/13/58	50	11	4	4
11/20/58	49*	20	4	4
12/9/58	49	42	4	4
12/29/58	48*	50	4	4

* One dead.
Key: BR, Barred Plymouth Rocks; WL, White Leghorns; RIR, Rhode Island Reds; W White Plymouth Rocks.
Group I birds were received on 9/30/58 and Group II birds were added on that date.

ine the CRD status of breeding flocks. Results reveal that most flocks are rologically positive for CRD. Three-year testing results reveal 6 flocks negave and 74 positive in 1956, 7 negative and 70 positive in 1957, and 10 negative d 63 positive in 1958. The majority of the positive flocks were tested by ndom-sample method; in many the entire sample was positive, indicating at in many flocks approximately 100 per cent of the birds were serologically sitive. The status of the negative flocks was based, for the most part, on l the birds tested in the flock. A few negative flocks were consistently negave over a 3-year period. Furthermore, the histories of these flocks revealed o evidence of CRD, and no new stock had been introduced.

In one commercial breeding flock CRD-free progeny has been developed by rocess of serologic testing and removal of infected birds. The testing results or this flock have been described in a previous publication.[12] A study of the eactors detected in the various pens of birds revealed interesting results (TABLE 5). At the laboratory the reacting birds were serologically tested, and nasal nd tracheal swabs were cultured in media. The cultures were tested for emagglutination and carbohydrate fermentation, and were inoculated into mbryonating eggs and birds. The findings reveal that birds giving a doubtful r a weak agglutination reaction are less likely to yield the CRD agent than re the stronger reactors. The carbohydrate method appeared to be the most ensitive test in detecting the CRD agent. This was followed by embryo- and ird-inoculation methods. With the limited amount of data it is impossible o recommend a procedure for testing CRD reactors that may be considered eliable. The establishment of CRD-free flocks with a serologic method has evealed several major obstacles. It appears that many flocks become serologially positive during the late growing stage (3 to 5 months of age). Flocks ested at a young age (1 to 3 months) may be partially or completely serologi-

Table 4

Contact Transmission of CRD Among Chickens as Determined by the Agglutination Test

Date of test	Positive	Negative contact			
	Group I	Group II	Group III		
	50 BR hens No. positive	25 RIR hens No. positive	7 RIR males No. positive	17 RIR pullets No. positive	7 WR pullets No. positive
10/7/58	50	0	0	0	0
10/23/58	50	1	0	0	0
11/6/58	50	1	2	6	0
11/20/58	49*	2	2	8	0
12/9/58	49	13	3	10	1
12/16/58	49	22	5	13	7
12/29/58	49	25	6	15	7

* One dead.

Key: BR, Barred Plymouth Rocks; RIR, Rhode Island Reds; WR, White Plymouth ocks.

Groups I and III, received 9/30/58 and placed in the same pen.

Group II, received 10/6/58 and added to Groups I and III.

cally negative. However, in reality such flocks appear to contain infected birds that under some stress may later become active spreaders of the disease. Once the infection begins to spread in a flock, the agglutination test is of little or no value in eliminating the infection because of its rapid and persistent dissemination through the flock. Once a flock has been found free of reactors, its progeny also will be free if reared in an environment free of PPLO. Also,

Table 5

Serologic and Cultural Studies of PPLO Reactors Obtained from a Commercial Flock

Pen No.	No. birds examined	Number of reactors by degree of reactions											Results of various tests					
													No. birds positive					Total birds positive
		First test					Final test						Cultures			Emb.	Bird	
		±	1	2	3	4	0	±	1	2	3	4	A	B	C			
I	9	6	3	0	0	0	4	1	2	0	1	1	1	0	1	0	NT	1
XI	14	9	1	2	2	0	2	4	0	3	1	4	2	0	4	3	3	4
XII	13	4	1	6	2	0	2	3	0	1	0	7	1	0	3	2	2	4
XX	10	0	0	2	3	5	0	0	1	0	1	8	7	1	10	10	6	10

Key: A, agar culture; B, hemagglutination test; C, carbohydrate studies; NT, not tested; Emb., embryonating egg.

Table 6

Re-exposure with the CRD Agent of Hens That Survived a Natural Outbreak of the Disease

Groups	No. birds	Number of birds positive				
		Pre-exposure	Postexposure			
		Serology	Serology	Symptoms	Lesions	Culture
A	9	9	9	0	0	1
B	7	7	7	0	0	0
C	10	0	10	6	10	10

Key: A, surviving birds, inoculated; B, surviving birds, noninoculated; C, susceptibl birds, inoculated.

Inoculum, 595-4 embryo material; route, right sinus and trachea; dose, 0.5 ml.; duratio of experiment, 4 weeks.

such stock appeared to withstand the vaccination stress that so often is responsible for prolonged and serious respiratory signs in CRD-infected stock. A practical method for the establishment of CRD-free stock has not been developed thus far, but it is hoped that further investigation may prove fruitful i this regard.

Other means of controlling or preventing disease, such as drug therapy o immunization, have given very little promise. Immunity studies showed that birds that have experienced the disease and no longer manifest clinical signs may be partially refractory to re-exposure to the CRD agent, while susceptibl irds inoculated with the same inoculum will show evidence of the disease TABLE 6). In order to protect young flocks against the disease, it would be ecessary to immunize the chicks at approximately 4 weeks of age. Whether mmunity sufficient to give adequate protection against the disease for the roductive life of the bird could be induced remains to be determined. This spect of the disease should be investigated.

Summary

The disease entities, CRD in chickens and IS in turkeys, are recognized as eing caused by a common agent designated as one of the PPLO. The wide istribution and economic importance of these disturbances have been stressed. he significance of egg transmission of the agent has been emphasized. The alient clinical signs, the pathological changes, and immunological features ave been discussed. Methods of control for the disease have been evaluated.

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PATHOLOGY OF PPLO AND OTHER AGENTS IN CHICKEN EMBRYOS

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The chicken embryo has been used for the past quarter of a century for studies in embryology, experimental biology, and medicine and, more recently, in the production of human and veterinary biologicals. It has been a very useful tool in research, particularly for the isolation of new viruses. It has been used extensively in the study of avian pathology, perhaps because avian disease agents grow readily in the embryonating chicken egg. As data on the effect of avian agents in the chicken embryo accumulated, practical uses evolved, such as diagnostic serum-neutralization tests for such specific chicken diseases as Newcastle and infectious bronchitis. This has resulted in the use by almost every important poultry research and diagnostic laboratory of thousands of dozens of eggs each year. Many tests are based on end points of living or dead embryos, and frequently the embryo never is examined grossly or microscopically. It is now well known that many chemicals, vitamins, viruses, bacteria, fungi, and other agents are responsible for mortality and extensive pathology, and it becomes increasingly important to have a disease-free source of eggs and to ascertain that injected material be known and free from spurious chemicals or other products that might affect the embryo. A clean flock for the source of eggs is essential, as was pointed out by Cottral,[1] who stated that the following agents were transmitted through the hen's egg: avian lymphomatosis, avian encephalomyelitis, Newcastle disease, infectious sinusitis (turkeys), psittacosis (parakeets), *Salmonella pullorum*, *Mycobacterium avium*, *Shigella gallinarum*, and other *Salmonella* species. It is absolutely necessary that chicken eggs for experimental work be obtained from well-managed flocks, preferably from closed flocks managed by those familiar with poultry diseases. Good nutrition of the egg-supply flock is critical, as shown by Romanoff and Bauernfeind.[2] When a deficiency of riboflavin existed in hens, seven eighths of the resulting embryos died before hatching. Eleven per cent of these died at 13 to 14 days' incubation. The embryos were dwarfed. Such a situation would seriously mislead a researcher who depended on a mortality at 13 to 14 days' incubation. Sanitary handling, good storage, and incubator hygiene to avoid the possibility of accidentally contaminating the eggs are important.

Vitamins and Chemicals

Hansborough[3] found that varying quantities of vitamins A and D or their precursors had a toxic effect when injected into 24-hour chicken embryos. The typical effects observed were: embryonic axis shorter than normal, hearts with a sigmoid curve or amorphous mass, absence of cranial and cervical flexures, omphalocephaly, or absence of amnion, chorion, and allantois. Cravens[4] stated that the analogue of nicotinic acid, 3-acetylpyridine, in sublethal doses (less than 600 μg./egg) injected into the yolk sac of 4-day-old embryos caused undersized, deformed legs and a generalized edema at the body surface.

Gentry[5] studied the toxicity of Achromycin·HCl, Aureomycin·HCl, Terramycin·HCl, Chloromycetin, neomycin, bacitracin, penicillin G sodium, dihydrostreptomycin sulfate, and furazolidone. He found the embryo LD_{50} varied greatly, depending on the route of inoculation. Ten-day-old embryos were all killed during the first 6 days postinoculation and showed lesions of edema and hemorrhage. Apparently, histological studies were not conducted. These studies are important because the antibiotics used were those that many laboratories use to reduce bacterial contamination when isolating viruses. By knowing the toxic dose the problem of nonspecific embryo mortality can be eliminated.

Research by Ridgway and Karnofsky[6] showed the toxicity of fifty-four elements in various salts. The nine outstanding elements when injected by yolk sac or chorioallantoic membrane (CAM) and the most specific lesions were: (1), (2) thallium and chromium—achondroplasia; (3) lead and cobalt—brain softening, hydrocephalus, hemorrhage, and edematous enlarged liver; (4) boron—pale and edematous, feather inhibition; (6) arsenic and rhodium—stunted, micromelia, abdominal edema; (8) barium—inhibition of toe growth; and (9) selenium—bizarre abnormalities, stunting.

Bacteria, Viruses, Rickettsiae, and Fungi

The chicken embryo has been used in medicine extensively since Goodpasture[7] discussed its various uses in 1933. Cox[8] was the first to show that rickettsiae of all types could be propagated successfully in the yolk sac. Other reports by Cox[9] and Buddingh[10,11] gave the detailed pathology with various agents.

Inclusion bodies of the following viruses can be detected on the chorioallantois (CA) when injected with psittacosis, vaccinia, lymphogranuloma venereum, herpes simplex, equine encephalomyelitis, fowlpox, ectromelia, Kikuth's canary virus, laryngotracheitis of fowls, and Pacheco's parrot disease. The predominant lesion in the CA of 10- to 14-day-old fowlpox-infected embryos was a hyperplasia and hypertrophy of that membrane. Pocklike eruptions were found on the ectodermal epithelium. Typical intracytoplasmic inclusions were noted in almost every cell. Little necrosis was observed, and a septicemia of the embryo with this virus rarely occurs. In contrast, the vaccinia virus when inoculated onto the CA of 12- to 14-day embryos produced a pocklike lesion on the ectodermal epithelium, and those cells became vacuolated within 24 hours with ulceration; necrosis and intracytoplasmic inclusions followed. Acute inflammation of the mesodermal layer and an endodermal infection were not uncommon. Variola virus, when inoculated onto the CAM of a 10- to 14-day-old embryo, produced a pocklike eruption in 48 to 72 hours. The pocks consisted of focal proliferations of ectodermal epithelial cells. Cytoplasmic inclusions with necrosis of the cells and ulceration of the nucleus were characteristic. A mild inflammatory reaction involved the mesoderm. Herpes simplex virus produced a pocklike eruption in 48 to 72 hours on the CAM. The focal lesions presented typical type-A intranuclear inclusion bodies. The virus spreads readily to the blood stream, and an encephalitis with focal areas of neuronal destruction, and intranuclear inclusion in the heart, liver, spleen, kidneys, striated muscle, and vascular endothelium results. Dawson[12] adapted the rabies virus to 10- to 14-day-old embryos by direct intracerebral inoculation. A grossly visible symmetrical hydrocephalus resulted, as well as other brain lesions of hydromyelia, encephalomalacia, and myelomalacia. An inflammatory reaction consisting chiefly of large mononuclears within the central nervous system (CNS) was common. Outside the CNS large collections of lymphocytes surrounded affected dorsal root ganglia, sympathetic ganglia, and intrinsic ganglia of the cytoplasm of the neurons, the ependymal epithelium, and the retinal cells; peripheral ganglia were found six to seven days post-inoculation. The infectious bronchitis virus of domestic chickens has been identified by the use of chicken embryos. Several workers[13-15] noted a dwarfing of embryos that were infected with the virus. Fabricant[16] confirmed these findings with 116 strains on more than 6000 embryos and noted that, in addition to the dwarfing, the embryo usually was curled tightly in a ball-like form. This was explained as a bending of the longitudinal axis of the embryo around its ventral surface. Loomis *et al.*[17] studied the macroscopic and microscopic pathology of the chicken embryo of 8 serial passages of a field strain of the virus inoculated into the allantoic sac. The embryos showed a grossly thinner CAM and a thickened dry fibrotic amniotic membrane with less than a normal amount of amniotic fluid. They were one half their normal size, with feather development sparse and with dermal petechiae. The livers were hemorrhagic and icteric and contained necrotic foci; the kidneys, swollen with necrotic foci; lungs small, pale, viscid with a serous exudate; heart, one half normal size; and the spleen, enlarged. The micropathology showed an excessive proliferation of the mesodermal and ectodermal cells, as well as edema of the CAM. The amniotic membrane was edematous. The liver showed hemorrhage, necrosis, and abscess formation. Other lesions included pneumonia with serous exudation, interstitial nephritis and necrosis, splenic congestion, and capillary congestion of the brain.

DuBose *et al.*[18] isolated a virus from quail that, when injected into the CA cavity of 10-day-old embryos, produced a dwarfing, thickening of the amniotic sac, engulfment of the embryo by the yolk sac, and mortality.

Yates and Fry[19] described an agent, thought to be a virus, that they isolated by inoculation of the allantoic cavity with material from the lymph node of a cow. In 7-day-old embryos this agent produced dwarfing and mortality. Histological studies failed to show any definite lesions.

Histological studies of the effects of several avian viruses on chicken embryos conducted by Jungherr *et al.* at the University of Connecticut, Storrs, Conn., will be described elsewhere in this monograph.

Bacteria and fungi are propagated most commonly in the chicken embryo for production of large quantities of the organisms, but not by this method for differentiation purposes, although this may have possibilities. There is some evidence that certain organisms have a predilection for tissues similar to those infected during the natural course of the disease. Goodpasture and Anderson[20] studied the following microorganisms on the CA of 12- to 14-day-old embryos, *Staphylococcus aureus*, *Streptococcus hemolyticus*, *Str. viridans*, *Eberthella typhosa*, *Aerobacter aerogenes*, *Corynebacterium diphtheriae*, and *Brucella abortus*, and found that the characteristic lesion resembled that found in the human disease.

E. typhosa was capable of invading the cytoplasm of the entodermal epithelial cells of the membrane, multiplying intracellularly, and forming small intracytoplasmic colonies. *Hemophilus influenzae* inoculated onto the CA of 11- to 12-day embryos produced a mild inflammatory response, but a septicemia resulted, with the development of a meningitis ependymitis and focal cerebral hemorrhages. *H. pertussis* produced an acute inflammatory reaction, local hemorrhage, and necrosis of the CA that, by the fifteenth day of incubation, resulted in a midzonal necrosis of the tracheal and bronchial epithelial lining; sometimes pneumonitis resulted. Buddingh[11] reported that the meningococci inoculated on the CA of 12- to 15-day-old embryos produced vascular injury such as petechial hemorrhages, extensive extravasations, and bacterial thrombi. Inoculation into the amnion resulted in a pharyngitis and paranasal sinusitis. Buddingh and Womack[21] showed that *Pasteurella tularensis* was highly pathogenic when inoculated onto the CA, within 80 hours invading the ectodermal epithelium and later causing focal necrosis of myocardium, liver, spleen, and kidneys. The plague bacillus is also highly lethal for the embryo and multiplies in the extracellular fluids and localizes in the liver. *B. suis* invaded the ectodermal epithelium, mesodermal fibroblasts, and vascular endothelium, whereas *B. melitensis* restricted its intracellular invasion to the ectodermal epithelium. *C. diphtheriae*, when inoculated into the amniotic fluid of 12- to 15-day-old embryos, gained entrance to the respiratory tract and mucous membrane, and developed as does the human disease. Necrosis of the epithelial lining results. *Streptobacillus moniliformis* produced a purulent arthritis when inoculated onto the CA of 12- to 14-day-old embryos.

Moore[22] studied various pathogenic and nonpathogenic fungi on the CA and noted that the inflammatory reactions were similar to those of the natural disease. Norris *et al.*[23] described the lesions produced by *Candida albicans* on the CAM of 11-day-old embryos. If the chick was not overwhelmed quickly by the infection, there were, at first, focal areas of liquefaction necrosis associated with hemorrhage and scanty granulocytic infiltration. As the lesions became older the granulocytic infiltration became greater, and larger numbers of mononuclear phagocytes and multinucleated giant cells, accompanied by proliferating fibroblasts, surrounded the focal necrosis.

Pleuropneumonialike Organisms (PPLO)

Chronic respiratory disease (CRD) of chickens and infectious sinusitis of turkeys have been thought to have an identical etiological agent.

In 1948 Delaplane[24] reported that 7-day-old embryos were preferable for the propagation of the CRD-producing agent. Gross embryo lesions consisted of hemorrhage of the liver, kidneys, and brain, whitish circumscribed foci on the CA, purulent inflammation of the joints, and brain hemorrhage. Van Roekel *et al.*[25] observed that gross lesions in chicken embryos consisted of stunting, edema, congestion, hemorrhage, and abscess (joints) as well as congestion, necrosis, and enlargement of kidneys and livers. Hitchner[26] cultivated the turkey sinusitis agent on CAM of chicken embryos and observed white 2-mm nodules on the membrane. Other gross lesions in the embryo were dark green-colored livers and granular urates in the allantoic sac. Pipped dead embryos showed complete consolidation of both lungs and caseous casts in the bronchi

As early as 1943 Delaplane and Stuart[27] isolated an agent that was later thought to be the cause of CRD. In 10-day-old embryos the gross lesions were extensive hemorrhages of the liver and kidneys. Some embryos showed whitish circumscribed foci on the CAM, enlarged and congested kidneys, and retention of urates.

Chute[28,29] and Chute and Cole[30] studied 9 chicken and 4 turkey strains of an agent isolated from chickens with CRD and turkey sinusitis, respectively.* The strains were diluted in nutrient broth 10^{-1} through 10^{-8}, and each 7-day-old embryo received 0.2 ml. of inoculum via yolk-sac route. Fourteen control eggs were used with each titration. The eggs were obtained from a White Leghorn flock that had not shown any respiratory disease. The eggs were candled daily through hatching and, if not dead prior to 21 days, were removed from the incubator and chilled in the refrigerator 2 hours prior to examination. All embryos were examined by transferring one loopful of yolk material to a tube of thioglycollate (BBL135) broth to test for contamination with aerobes or anaerobes. No contamination was experienced at any time. The third toe or middle toe of all embryos 12 days old or older was measured. This measurement was made in millimeters from the middle of the metatarsal joint to the tip of the toe as described by Hamilton[31] and used as a criterion for dwarfing. The yolk stalk was smeared and Giemsa-stained. After thorough gross examination the remainder of the embryo was fixed in 10 per cent formal-saline and used for histological sections. Routine hematoxylin and eosin stains of the embryos were made, except in special cases in which Giemsa's, Unna's, polychrome methylene blue, Machiavello's, Victoria Blue R, Castaneda's, Wilder's silver oxide, Bodian's, and von Kossa's stains were used. TABLE 1 gives a summary of the history, embryolethal dose, number of serial passages, embryomortality pattern, and special features of each PPLO strain.

The gross pathology of the embryos was observed by periods. Embryos that died between 8 to 14 days of incubation showed extensive hemorrhage and edema. Ecchymotic hemorrhages were noted frequently in the skin of the head, neck, and ventral thoracic region. Sparse feathering, thickened, opaque CAM, and generalized edema were common. Embryos that died between 14 to 21 days of incubation presented hemorrhage and edema. Definite dwarfing, substantiated by middle toe measurements, was common. CAMs were thickened, opaque, dry, and closely adherent to the embryo. The leg and wing joints were puffy; white and acellular homogenous fluid poured out when sectioned. White, hard, circumscribed granulomas were noted in the higher passaged strains. Many livers were enlarged and hyperemic, while others had a granular surface and contained small white necrotic foci. Pleuritis and renal hemorrhage were noted.

The histopathology of embryos that died at 9 to 14 days of age showed myocardial hemorrhages and passive hyperemia. The skin and muscles in the region of the vertebrae were edematous, as evidenced by pink homogenous material in the interstices. There was a disintegration of the spinal cord and a lymphocytic infiltration of the splanchnic ganglia and lungs. The livers presented general passive congestion and hepatic cell necrosis around many central veins. The sinusoids contained many lymphocytes. The kidneys showed mild to severe interstitial hemorrhage. The embryonic brain showed diffuse edema and gliosis. Embryos that died at 15 to 21 days of age presented myocarditis, hyperemia, edema, and lymphocytic infiltration throughout the myocardium. Endocarditis, fibrinous pericarditis with necrosis, and epicarditis were seen. Liver lesions were characterized by focal necrobiosis, minute to large foci of lymphocytes, parenchymatous degeneration, and hyperemia. Periphlebitis was observed. Heterophils infiltrated the perivascular spaces and walls of the veins. Hemorrhage filled the tertiary bronchi and alveoli. Periarteriolitis, arteritis, and periarteritis were frequent lesions of the pulmonary vessels. The perivascular spaces, adventitia, and tunica media of most arterioles and arteries were infiltrated with heterophils. There were neuronal degeneration, edema, diffuse gliosis of the brain, and necrosis of the joint cavities. The CAM showed hyperplasia of the ectodermal layer, necrosis, hyperemia, and hemorrhage. Giemsa-stained sections often revealed several large, round, pale-blue epithelial cells filled with 8 to 12 round, discrete, dark, purple-staining organisms 0.5 to 0.9 μ in size. These were considered to be PPLO. The circumscribed yellow-to-white raised nodules, varying in size from 1 to 4 mm. in diameter, found under the skin of the tibial and femoral portions of the legs, on the hock, toe, and wing joints, and in the gizzard were found to be granulomas. Microscopically, the nodules were composed of a necrotic center sur-

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* The strains were received in yolk culture from Henry Van Roekel, University of Massachusetts, Amherst, Mass.; Floyd Markham, Lederle Laboratories, Pearl River, N.Y.; and B. S. Pomeroy, University of Minnesota, St. Paul, Minn.

TABLE 1

HISTORY AND CHARACTERISTICS OF STRAINS OF PPLO STUDIED

Strain	Source	Species origin	No. of passages	MLD (per cent)	Embryo mortality pattern (deaths at days' incubation)	Special features
A	Van Roekel	chicken	4	44.4 @ 10^{-3} dil.	9, 20	Few lesions
B	Van Roekel	chicken	1	50 @ 10^{-1} dil.	17, 20	Low titer, few lesions
C	Van Roekel	chicken	2	50 @ 10^{-4} dil.	9, 11, 13, 16, 19, 20	High titer, many lesions
D	Van Roekel	chicken	21	43.7 @ 10^{-3} dil.	14, 15, 17, 18, 30	Many lesions
E	Van Roekel	chicken	1	44.4 @ 10^{-1} dil.	16, 20	Low pathogenicity for embryos
F	Van Roekel	chicken	8	50 @ 10^{-1} dil.	14, 16, 20	Low titer
I	Markham No. 1136	chicken	82	45 @ 10^{-3} dil.	10, 17, 19	Strain producing granulomas
K	Markham (W.T.)	chicken	41	50 @ 10^{-4} dil.	9, 10, 13, 15, 16, 19	Granulomas produced
N	Pomeroy (Y)	chicken	—	.66 @ 10^{-1} dil.	9, 10, 11, 17, 19, 20	Low pathogenicity for embryos
G	Van Roekel	turkey	9	52.1 @ 10^{-4} dil.	9, 10, 11, 12, 13, 14, 15, 19, 20	High titer
J	Markham No. 2037	turkey	88	45 @ 10^{-3} dil.	9, 10, 12, 15, 17, 20	
L	Pomeroy "B"	turkey	—	50 @ 10^{-2} dil.	10, 11, 12, 16, 18	No deaths 19 or 20 days
M	Pomeroy "I"	turkey	—	45 @ 10^{-5} dil.	9, 10, 12, 13, 14, 17, 18, 19, 20	Very pathogenic for embryos

rounded by epitheloid cells that had coalesced to form giant cells containing from 2 to 27 nuclei.

Adler and Yamamoto[32] described briefly the pathology of a PPLO isolated from turkeys inoculated onto the CAM of 6- to 8-day-old embryos and noted that pocklike lesions developed.

Moulton and Adler[33] described in detail the joint pathology of an eighth passaged chicken-embryo PPLO inoculated into the yolk sacs of 8-day-old embryos. They observed a gross tibiofemoral joint involvement, as well as extensive cutaneous hemorrhages in the pectoral region. They divided the pathology into stages and generally observed necrosis of the synovial cell layer and later of the articular cartilage. Necrosis preceded cellular invasion of the joint cavity. The joint capsule was infiltrated by mononuclear cells by the third day postinoculation, but the joint cavity was free of cells until the sixth day. The vascular and cellular components of the inflammation subsided by the eighth day postinoculation, and foci of mononuclear cells in the periarticular sites were encapsulated by connective tissue. In a few cases, there was foreign-body granulomatous tissue.

Van Roekel *et al.*[34] studied 127 chicken embryos microscopically during a qualitative survey of the lesions produced by the CRD agent. The embryos ranged from 16 to 20 days of age. They found that the lung involvement was characterized by perivascular infiltration of inflammatory cells, chiefly mononuclear, septal edema, and cellular exudation. The air sacs showed mononuclear cell infiltration and focal epithelial hyperplasia. The trachea presented a hyperplastic epithelium and necrotic cellular debris. The kidney showed a tubular degeneration, hemorrhage, and a mononuclear cell infiltration. Perivascular infiltrations, necrobiosis of hepatic cords, and massive necrosis of the peripheral areas of the lobes were found commonly. Suppurative arthritis characterized by a heterophilic exudate and necrosis was observed in the joint cavities. The heart showed subepicardial and myocardial infiltration by mononuclear cells. The work may be summarized by stating that the CRD agent produced vascular lesions, both inflammatory and degenerative, as well as an exudative involvement of the respiratory tract and subcutaneous necrotic foci.

Jungherr and Luginbuhl[35] stated that in embryos the lesions are entirely granulomatous and are confined to the lung, and that the lumina of tertiary bronchi are filled with hemorrhagic debris and often girded by giant cells.

Discussion

The chicken embryo has been shown to be an invaluable tool in human and veterinary research, as well as in other areas of biological study. Being a completely sterile unit with a specific metabolic and enzyme system, it offers an unlimited field of study. It is hoped that this paper will stimulate research in the use of chicken embryos as the criteria for the identification and pathological response of PPLO and other agents in this medium.

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BASIC PATHOLOGICAL RESPONSES TO AVIAN PPLO IN CHICKENS AND CHICKEN EMBRYOS*

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The basic pathological responses of susceptible host tissue are an integral part of the over-all characterization of an infectious agent. With the recent advances in the cultural and serologic differentiation of avian pleuropneumonialike organisms (PPLO) by Yamamoto and Adler (1958*a*, *b*) and Chu (1958), and in view of the impending designation of pathogenic species as *Mycoplasma gallisepticum* by Edward (1959), the definition of the basic pathological responses gains in both academic and practical importance. The available information pertains almost exclusively to known pathogenic strains, while further work is necessary to elucidate such potentialities of other recognized types. It may be premature to assume a sharp split of avian PPLO into pathogenic and nonpathogenic species, especially since it is known that (1) the field syndrome of air-sac disease is often complicated by the interaction of several infectious agents; (2) experimentally induced infections do not present gross differential features; (3) clinically normal birds may present specific lesions on histopathological examination; (4) PPLO strains of known pathogenicity may lose this attribute after prolonged culture passage; and (5) flocks known to be PPLO-free usually fail to yield PPLO of any type.

The pathological concept to be presented is based upon replicate (at least 3 times) histopathological examinations of chickens and chicken embryos inoculated with pathogenic, culture-propagated, hemagglutinating PPLO, and sacrificed at 2- to 3-day intervals postinoculation. All birds and embryos used for this purpose were obtained from parent stock known to be PPLO-free and maintained in isolation.

Since pathogenic strains from different avian sources elicited similar basic responses in inoculated birds (Jungherr *et al.*, 1954), the principal observations are presented with reference to organs or systems affected.

Respiratory Tract

Trachea. After intratracheal inoculation (0.5 ml. of a young broth culture of PPLO, thirtieth to sixtieth passage) the tracheal mucosa remains essentially normal for 9 to 12 days except for distention of the intraepithelial glands. Thereafter the typical changes of catarrhal tracheitis are present. The mucosa is swollen to 5 times its normal thickness and the epithelial cells are deciliated, hypertrophied, and closely spaced, with consequent compression or elongation of intraepithelial glands. The propria displays proliferation of mononuclear elements, chiefly histiocytes and lymphocytes, with a tendency toward a follicular arrangement. Within 21 to 24 days postinoculation, the mucosa reverts

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to the normal architecture, but scattered, occasionally bulging, lymphofollicular areas may remain in the propria.

The pathological alterations, even at their height, differ markedly from those in field cases of air-sac disease. The latter lesions are characterized by intense swelling of the mucosa, with a flat, garlandlike, vacuolated epithelium and dense mononuclear infiltration of the propria (Jungherr *et al.*, 1953). This field picture, however, can be reproduced experimentally by exposing birds first to PPLO and, one week later, to infectious bronchitis virus.

The lesions resulting from uncomplicated PPLO infection differ also in time of development and essential characteristics from those induced by Newcastle disease, infectious bronchitis, and laryngotracheitis viruses (Jungherr and Luginbuhl, 1952*a* and *b*).

Lungs. Hyperplasia of lymphoid follicles beneath the epithelium of primary and secondary bronchi is a common nonspecific finding in avian respiratory diseases. Lesions characteristic for PPLO infection develop about the same time as the tracheal lesions, but outlast the latter. They are represented by scattered lymphofollicular structures in the walls of the respiratory tubules (in the terminology of McLeod and Wagen, 1939), often jutting out into the lumen of the tertiary bronchi. Giant-cell granulomas are rare in comparison with field cases, which may be accompanied by focal heterophil exudation.

The air sacs become implicated in the pathological process at an early stage of the disease and reflect the basic changes in the lung. Lymphofollicular areas form bulges in the thin stroma in a rosarylike fashion. It is only during the late stages, when secondary agents—particularly coliform bacteria—play a role, that these changes are accompanied by massive inflammatory edema and intense heterophilic cellular exudation.

Infraorbital sinus. After intrasinusal injection, the normally thin mucosa reacts in a highly specific manner within 3 to 7 days. While the epithelium of the injected sinus presents essentially the catarrhal changes described for the trachea, the propria exhibits the highest reactivity. The normally cell-poor propria, in contrast to the relatively cell-rich corresponding layer of the trachea, is either diffusely infiltrated by mononuclear cells or has massive, crowded, lymphofollicular structures. Combinations occur, but the diffuse type prevails in turkeys. The mucosa of the homolateral turbinates is likewise involved in the catarrhal process. Heterophil and mucous exudation, responsible for the clinical sinusitis, occurs later. It is not entirely safe to judge the results of intrasinusal injection solely on a clinical basis. Bilateral histopathological examination of the nasal cavities provides a "built-in" control in the uninoculated sinus, but has the disadvantage of requiring decalcification.

The Visceral Organs

Liver, spleen, heart, and kidneys do not disclose significant lesions. This concept is at variance with previous observations on a turkey air-sac strain (Minard and Jungherr, 1944) that produced splenomegaly and multiple reticular cell foci in the liver (Jungherr, 1949). From the facts that the original turkey specimen had tarsal inflammatory lesions (not appreciated at the time), in addition to severe aerosacculitis, and that known strains of synovitis virus produce splenomegaly and such hepatic reticular-cell foci in chickens (Sevoian *et al.*, 1957) and chicken embryos (Casorso and Jungherr, 1959), it is very probable that the original strain was a mixture of PPLO and synovitis virus. In retrospect, the early observations are a remarkable demonstration of the etiologic significance of basic pathological responses.

In delayed experimental PPLO infections, particularly in field cases, the covering membranes of the visceral organs are likely to give evidence of purulent serositis associated with granulomatous foci very similar to the late changes in the air sacs and attributable to the interaction of secondary invaders.

The Central Nervous System

The original turkey air-sac strain (1c) had the remarkable ability of inducing, after parenteral injection, clinical paresis and severe endarteritis in the brain and meninges, accompanied by secondary malacic foci and associated glial mobilization. After years of failure to observe similar PPLO strains, there has come unexpected confirmation of the occasional neurotropism of PPLO from the report of Cordy and Adler (1957). Their strain actually was isolated from the brain of a turkey, maintained its encephalitogenic properties through many passages, and produced lesions of a similar basic pattern.

The Eye

Inoculation into the anterior chamber of the eye (Jungherr *et al.*, 1953) with 0.02 ml. of young broth cultures of pathogenic (chicken red cell-agglutinating) PPLO strains produces a progressive panophthalmitis within 7 to 10 days. Nonhemagglutinating strains fail to do so, but may induce slight nonspecific reactions, as observed by Burmester (1952) after the injection of tumor filtrates. The inoculation is made with a 27-gauge needle from the outer canthus and parallel to the iris, in order to avoid trauma of the lens.

A clinical "take" may be expressed by lacrimation, bulbar swelling, corneal opacity, or hypopyon. A histological section, preferably through the level of the pecten, discloses inflammatory involvement of most internal structures. At times the bulbar conjunctiva shows lymphofollicular reaction. Cornea and anterior chamber are only mildly involved, while the corneoscleral junction, iris, and ciliary body present intense diffuse infiltration with mononuclear cells. The process diminishes in intensity along the chorioid, and often stops abruptly at the ora serrata. The lens is never involved unless injured in the course of injection. While the aqueous humor may contain seemingly loose debris and inflammatory cells, in a high percentage of the cases the pecten is thickened owing to mononuclear infiltration of its capillary plexus.

In comparable tests with other agents such as those of Newcastle disease and infectious bronchitis virus, the inoculation either killed the birds or caused a rapidly developing, mild, short-lived inflammation of a nonspecific character (Jungherr *et al.*, 1953). Thus the eye seems to constitute a tissue highly susceptible to the pathogenic action of avian PPLO and seems to react in a characteristic if not pathognomonic manner.

Chicken Embryo

In a histopathological study of chicken embryos that had died or had been killed by chilling after inoculation with avian PPLO, Chute and Cole (1954) found a variety of lesions such as widespread congestion and petechiation, focal necrobiosis and "lymphocytic" infiltrates in the liver, diffuse gliosis in the brain, heterophil perivasculitis, and late granulomas. From a similar study of chicken embryos in the late stages of incubation, Gray (1953) and Van Roekel *et al.* (1957) considered heterophil, perivascular infiltration, necrotic cellular plugs, and granulomas in the respiratory tract to be the characteristic alterations. In restudying this problem with infected embryos killed by exsanguination, Casorso and Jungherr (1959) found heterophil perivasculitis in the parenchymatous organs (especially the liver and, in the late stages, the lung) to be the only consistent pathological response. Some strains produced heterophil synovitis. The response was similar to that found with both hemagglutinating and nonhemagglutinating PPLO. The discrepancy between the response to the former and the responses reported in this paper may be explained by the facts that dead embryos were used (in which hypostatic phenomena confuse the picture), and that the infiltrating cells were designated lymphocytes, which occur only terminally in embryonic blood, according to Sugiyama (1926).

The pathological response in the chicken embryo, unlike that in the eye, can not be used for the differentiation of presumably pathogenic (hemagglutinating) and nonpathogenic (nonhemagglutinating) avian PPLO. The exclusive heterophilic response, in distinction to the basic mononuclear response in the adult, is ascribed to the fact that, throughout most of the embryonic life, the heterophil is the only leukocytic type available for inflammatory mobilization.

Discussion and Summary

The concept of the basic pathological response to avian PPLO set forth in this paper is in essential agreement with corresponding reports on the adult respiratory tract by Hitchner (1949), Johnson (1954), Gray (1953), and Van Roekel *et al.* (1957) and with reports on the central nervous system by Cordy and Adler (1957). Different observations in chicken embryos by Chute and Cole (1954) may be explained by the use of dead embryos and a variant interpretation of the infiltrating leukocytes. The formerly recognized reticular-cell foci in the liver (Jungherr, 1949) are now believed to be due to an admixture of avian infectious synovitic virus to the PPLO strain then employed.

Inoculation into the trachea of pathogenic (chicken red cell-hemagglutinating), culture-propagated, avian PPLO of low passage induces a catarrhal tracheitis accompanied by diffuse or follicular lymphoid infiltration of the propria. The lesions appear within 1 to 2 weeks and disappear in 3 to 4 weeks postinoculation. Superinfection with infectious bronchitis virus produces an intense, long-lasting mononucleocellular tracheitis, indistinguishable from that seen in the field air-sac syndrome. The lungs respond characteristically by the development of many intramural lymph follicles in the respiratory tubules of the tertiary bronchi. The thin air-sac stroma presents similar lymph follicles in a rosarylike arrangement. The visceral organs are not affected. Seropurulent exudation and granulomas in the respiratory tract, on the air sacs, and on serous membranes are probably caused by the interaction of secondary, often coliform, microbial invaders.

Inoculation of PPLO into the infraorbital sinus of chickens (and turkeys) brings about an intense, diffuse or follicular, lymphoid infiltration of the mucosal propria. Inoculation of the anterior chamber of the chicken eye induces a progressive panophthalmitis. The accompanying mononucleocellular infiltration involves especially the corneoscleral junction, iris, ciliary body, and pecten.

The eye of the chicken and the infraorbital sinus of the chicken (and turkey), in that order, represent the most sensitive tissues for the unequivocal evaluation of the pathogenicity of avian PPLO. Inoculation tests, in which trauma to the cavity linings is to be avoided, are best verified by histopathological examination ten days after injection.

The chicken embryo does not lend itself to differential pathogenicity tests, since it responds in a similar manner to both pathogenic and nonpathogenic avian PPLO.

The available knowledge pertains to known pathogenic avian PPLO of the general type classified by Edward as *Mycoplasma gallisepticum* elsewhere in these pages. Further work is necessary to elucidate the pathogenic potentialities of other avian PPLO types.

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DISCUSSION OF PART VII

A. W. Rodwell (*Animal Health Research Laboratory, Victoria, Australia*): I propose to add several comments to Piercy's paper on the antigenic structure of *Mycoplasma mycoides*. Galactan accounts for 10 per cent of the dry weight of the organisms. This material was isolated by the phenol extraction method of Westphal from the pellet obtained after freezing and thawing washed cells. It was freed from RNA by two methods: either by differential centrifugation in the Spinco model L or by using column chromatography. The preparations contained small and variable amounts of nitrogen, about 0.2 to 0.8 per cent, traces of phosphorus, less than 0.1 per cent, and about 2 per cent of ash.

After hydrolysis, about 85 to 89 per cent of the galactan was accounted for as reducing sugar calculated as anhydrous hexose. Galactose was the only sugar present. There is, therefore, a small residue that has not yet been characterized. It is not pyrogenic in rabbits, and it gives a single component after paper electrophoresis.

Serologic reactions of this material have been studied by Cottew (*Australian Vet. J.*, in press). It gives a precipitin reaction with pleuropneumonia antisera, and a single line in agar-gel diffusion tests. It is capable of sensitizing sheep, fowl, horse, or rabbit erythrocytes, so that they are agglutinated by pleuropneumonia antisera. The polysaccharide itself inhibits the agglutination of sensitized red cells by antisera. This indirect hemagglutination inhibition test is now being evaluated as another test to supplement or supplant the established complement fixation test, particularly as a diagnostic test to detect the chronic carrier animals.

Comment: It was interesting to note that J. G. Lecce found, associated with the L form or PPLO organism, a *Hemophilus* organism that required essentially the same growth factors as did the L form. If this is the case, perhaps some of these organisms that have not responded on the usual medium described by Smith and Morton could be derived from an organism that has special growth characteristics such as the *Hemophilus*, which requires coenzyme and the hematin factor. This may further explain organisms isolated from urethritis. It has been found that about 20 per cent of the gonococci require glutamine and about 0.2 per cent require cocarboxylase. If these exacting strains of gonococci give rise to L forms or PPLO forms with similar nutritional requirements, this fact may be a very important way of detecting them.

E. M. Lerner (*National Institutes of Health, Public Health Service, Bethesda, Md.*): I raise a morphologic point, one of morbid anatomy rather than bacterial morphology, about J. G. Lecce's paper on arthritic lesions, or so-called arthritis; I think it also pertains to W. C. Kuzell's paper on arthritis produced in rats with the L4 organism.

This point is raised, not to quibble over details, but because of the possible implication of this group of microorganisms in human diseases. Some of these diseases frequently are associated with joint lesions. Most of the lesions that have been presented and described as arthritis are similar to lesions that have been observed and occasionally produced with *Streptobacillus* in mice and rats and with L4. These are also generally similar to many of the so-called arthritic lesions produced in experimental animals by a number of bacterial species, in that they do not resemble human rheumatoid arthritis. Most of these, if not all, it would seem, start as a lesion of the bone, as a primary osteomyelitis, and extend into the surrounding joint tissues and involve the joint secondarily or, perhaps, even at the third stage. In contrast, rheumatoid arthritis involves the joint lining or the synovial membrane first, and often remains there without involvement of the surrounding joint tissues. The error frequently has been made of calling this arthritis in rats. When observed closely, involvement of the joints is seen, but this follows the involvement of the bone and of the soft tissues surrounding the joint. The joint is almost always the last item to be involved in the process. Therefore, this form of osteomyelitis should not be considered arthritis.

QUESTION: Is there any evidence or information regarding transmissibility of PPLO from animals to humans?

ERWIN JUNGHERR (*University of Connecticut, Storrs, Conn.*): This is a difficult question.

H. P. CHU (*School of Veterinary Medicine, Cambridge, England*): I wonder whether D. R. Cordy has made any serologic comparisons of his strains with the caprine pleuropneumonia and the *agalactiae* PPLO. I have isolated a caprine pleuropneumonia strain from a goat in turkey and, although the disease was not supposed to affect sheep naturally, I exposed a goat and a sheep experimentally, using an atomizer, and obtained some very interesting results. In the goat, PPLO produced a generalized pleuropneumonia; but in the sheep it produced only some localized lesions in the apical lobes of the lungs and the lymph nodes. In both goat and sheep a temperature reaction was obtained.

DONALD R. CORDY (*University of California, Berkeley, Calif.*): Several years ago Edward gave me some sera for serologic identification. The goat arthritis PPLO failed to agglutinate a contagious *agalactiae* antigen. Edward admitted that he did not value highly the caprine pleuropneumonia antigen he had sent.

It has been difficult to import some of these sera in recent times, and I gave the strain to the United States Department of Agriculture Laboratory at Plum Island, N.Y. I have not had any recent report on the strain.

This organism ferments glucose and a number of other carbohydrates. It liquefies inspissated serum and is hemolytic. It behaves very much like the caprine pleuropneumonia according to the criteria published by Edward, that is, it has not produced pneumonia either in the field or experimentally.

M. S. COVER (*University of Delaware, Newark, Del.*): J. G. Lecce mentioned five strains of an agent that causes tendovaginitis in chickens. Is this what has been called infectious synovitis for the last few years?

JAMES G. LECCE (*North Carolina State College, Raleigh, N.C.*): I believe that the two diseases are identical.

COMMENT: I propose to comment on E. M. Gianforte's reference to Gross's work. Gross produced CRD with *Escherichia coli* and PPLO strains isolated from field cases. The CRD condition produced by Gross apparently resembled that produced by Gianforte, except that Gross's work with aerosols produced lesions of greater severity than those described by Gianforte and quite similar to those shown in H. Van Roekel's excellent photographs of the CRD complex.

Julius Fabricant (*Cornell University, Ithaca, N.Y.*): On behalf of H. E. Adler and myself may I say that we are not quite satisfied with the current status of the avian species classification. Based on the criteria available, I find that there is a large group of strains already studied in the United States that does not fall into any of these categories.

Let us use just the first two criteria for separating these species: the forming of a film on the surface and the fermentation of glucose. *Gallinarum* is plus-minus; *iners* is minus-minus. The third group, the *gallisepticum*, is minus-plus. The largest group observed in California and New York is one that is plus-plus. We described these in our paper as the C-type strain, and this strain is so common that it deserves some consideration.

Erwin L. Jungherr: Would Edward exclude pathogenicity as a criterion there?

D. G. ff. Edward: I shall try to make matters clear. All I did was to examine 40 strains obtained from other laboratories in 1955. It is perhaps unfortunate that details of my findings have not been reported until now. Of the strains received in 1955, all except one of those, finally identified as *Mycoplasma gallisepticum*, came from Markham. I do not claim that all avian strains belong to the three species I have established, although the majority of strains I examined did. There were, however, a few strains that fermented carbohydrates, but differed in their properties from any of the three species. They were not classified further. I prepared an antiserum against one, but it did not react serologically with any other strain.

Erwin L. Jungherr: The two criteria, hemagglutinability and pathogenicity, are not considered in this classification.

H. P. Chu: I agree with Fabricant that all of the criteria must be considered. Previously I have described differences between the pathogenic and the so-called nonpathogenic strains. The hemagglutinin production could not be found in the nonpathogenic ones. I do not know whether Edward has looked into this. Also, I believe that the pathogenicity for chickens and turkeys should be tested with all strains isolated.

I agree with Jungherr that there may be some potential pathogenicity for the nonpathogenic strains. It is true that they cause a very similar mortality pattern of the embryos, but they differ significantly in their effect on the respiratory tracts of the embryos. The presence of exudate in sinus and air sacs, as described by Van Roekel, may be seen only in embryos infected with the pathogenic strains and not in those that died from the nonpathogenic strains. Jungherr also referred to the pathogenicity for chicken embryos, stating that there is no difference between the pathogenic and the nonpathogenic strains. I do not entirely agree. However, according to our discussion, the pathogenicity refers to the ability of a pleuropneumonialike organism by itself to cause CRD; it is on this basis that we separate the pathogenic PPLO from the nonpathogenic ones. Whether the latter are pathogenic in other respects or whether they may be an important secondary invader in other respiratory diseases is a different matter. All of the work described by Adler and myself points to the same thing: that the so-called pathogenic avian PPLO fit in with coccobacilliform bodies that Nelson found in 1935. In regard to taxonomy, credit should be given to Nelson for identifying the organism.

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